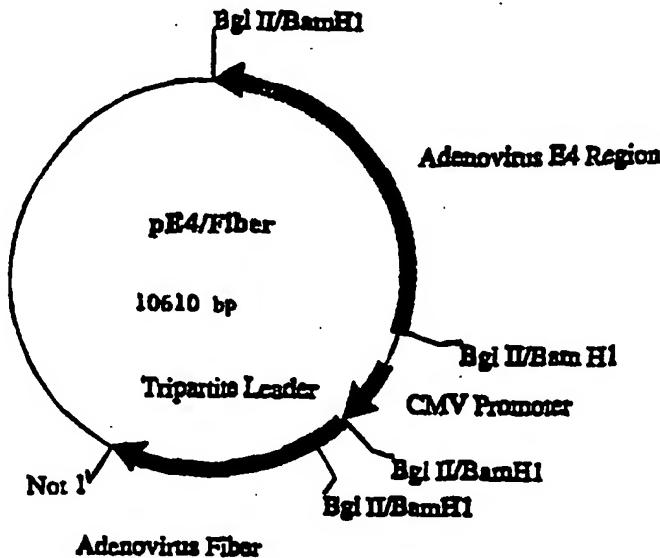




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(54) Title: PACKAGING CELL LINES FOR USE IN FACILITATING THE DEVELOPMENT OF HIGH-CAPACITY ADENOVIRAL VECTORS



(57) Abstract

The present invention relates to gene therapy, especially to adenovirus-based gene therapy, and related cell lines and compositions. In particular, novel packaging cell lines are disclosed, for use in facilitating the development of high-capacity vectors. The invention also discloses a variety of high-capacity adenovirus vectors and related compositions and kits including the disclosed cell lines and vectors. Finally, the invention discloses methods of preparing and using the disclosed vectors, cell lines and kits.

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PACKAGING CELL LINES FOR USE IN FACILITATING THE DEVELOPMENT OF HIGH-CAPACITY
ADENOVIRAL VECTORS

This invention was made with U.S government support under NIH Grant No. HL 54352. The government has certain rights in the invention.

The present invention relates to gene therapy, especially to adenovirus-based gene therapy. In particular, novel packaging cell lines are disclosed, for use in facilitating the development of high-capacity vectors. High-capacity adenovirus vectors are also disclosed herein, as are related compositions, kits, and methods of preparation and use of the disclosed vectors, cell lines and kits.

Enhanced transfer of DNA conjugates into cells has been achieved with adenovirus, a human DNA virus which readily infects epithelial cells (Horwitz, "Adenoviridae and their replication", in Virology, Fields and Knipe, eds., Raven Press, NY (1990) pp. 1679-1740).

Although adenovirus-mediated gene therapy represents an improved method of DNA transfer into cells, a potential limitation of this approach is that adenovirus replication results in disruption of the host cell. In addition, adenovirus also possesses oncogenic properties including the ability of one of its proteins to bind to tumor suppressor gene products. The use of so-called replication defective strains of adenovirus (which typically possess E1A and/or E1B deletions that render the virus unable to replicate in host cells) is in principle more suitable for *in vivo* therapy; however, the potential of co-infection of epithelial cells with wild-type strains of virus resulting in transactivation of the recombinant virus may represent a significant safety concern for *in vivo* applications. Furthermore, it is not yet known which recombinant adenoviruses are capable of integrating their genome into host cell DNA allowing for long-term stable expression of any foreign genes they may be transporting.

Another undesirable aspect of using intact or replication-competent adenovirus as a gene transfer means is that it is an oncogenic virus whose gene products are known to interfere with the function of host cell tumor suppressor proteins as well as immune recognition molecules, such as the major histocompatibility complex (MHC). In addition, pre-existing circulating antibodies to adenovirus may significantly reduce the efficiency of *in vivo* gene delivery. Lastly, only a foreign gene of 6 kilobases (kb) or less can be incorporated into the intact adenovirus genome for gene transfer experiments, whereas DNA segments of greater than 15 kb can be transferred using the methods of this invention.

In order to make Ad vectors more replication-incompetent, some investigators have attempted to construct recombinant Ad-derived vectors which have nearly all of their genome deleted, except for portions known to be required for packaging of virus particles. For example, helper-dependent vectors lacking all viral ORFs but including essential *cis* elements (the inverted terminal repeats -- ITRs -- and the contiguous packaging sequence) have been constructed, but the virions package less efficiently than the helper and package as multimers part of the time, which suggests that the virus may "want" to package a fuller DNA complement (see, e.g., Fisher, et al., Virology 217: 11-22 (1996)). Mitani et al. (PNAS USA 92: 3854-3858 (1995)) also describe a helper-dependent Ad vector that was apparently not completely replication-defective.

Amalfitano, et al. (PNAS USA 93: 3352-3356 (1996)) describe the construction of an Ad packaging cell lines that support the growth of E1- and polymerase-deleted Ad vectors, in an effort to block the replication of Ad vectors *in vivo*. Similarly, Armentano, et al. (Hum. Gene Ther. 6: 1343-53 (1995)) describes Ad vectors with most -- but not all -- of the E4 sequence deleted therefrom. However, since such a small amount of genetic material is deleted from the vectors, their ability to transport therapeutic sequences is rather limited.

In view of the aforementioned problems, the design and construction of the within-disclosed packaging cell lines and systems provides a novel and elegant solution, as described further herein. The use of the recombinant sequences and vectors of this invention to mediate the transfer of foreign genes into recipient cells both *in vitro* and *in vivo* overcomes the limitations of the above-described gene transfer systems. This invention utilizes recombinant constructs which duplicate the cell receptor binding and DNA delivery properties of intact adenovirus virions and thus represents an improved method for gene therapy as well as for antisense-based antiviral therapy.

In contrast to the disadvantages of using intact adenovirus, modified adenovirus vectors requiring a helper plasmid or virus, or so-called replication-deficient adenovirus, the use of recombinant adenovirus-derived vectors according to the present invention provides certain advantages for gene delivery. First, the Ad-derived vectors of the present invention possess all of the functional properties required for gene therapy including binding to epithelial cell receptors and penetration of endocytic vesicles. Therapeutic viral vectors of the present

invention may also be engineered to target the receptors of and achieve penetration of non-epithelial cells; means of engineering viral vectors to accomplish these ends are described in detail hereinbelow.

Second, the vectors of the present invention have deletions of substantial portions of the Ad genome, which not only limits the ability of the Ad-derived vectors to "spread" to other host cells or tissues, but allows significant amounts of "foreign" (or non-native) nucleic acids to be incorporated into the viral genome without interfering with the reproduction and packaging of the viral genome. Therefore, the vectors of the present invention are ideal for use in a wide variety of therapeutic applications.

Third, while the vectors disclosed herein are safe for use as therapeutic agents in the treatment of a variety of human afflictions, they do not require the presence of any "helpers" for propagation and packaging, largely because of the novel cell lines in which they are reproduced. Such cell lines -- referred to herein as packaging cell lines -- comprise yet another aspect of the invention.

To reduce the frequency of contamination with wild-type adenovirus, it is desirable to improve either the viral vector or the cell line to reduce the probability of recombination. For example, an adenovirus from a group with less homology to the group C viruses may be used to engineer recombinant viruses with little propensity for recombination with the Ad5 sequence in 293 cells. Similarly, an epithelial cell line -- 293 or another -- may be prepared according to within-disclosed methods which stably expresses adenovirus proteins or polypeptides from Ad3 and/or proteins or polypeptides from another non-group-C or group C serotype; such a cell line would be useful for supporting adenovirus-derived viral vectors bearing deletions of regulatory and/or structural genes, irrespective of the serotype from which such a vector was derived.

It is also contemplated that the constructs and methods of the present invention will support the design and engineering of chimeric viral vectors which express amino acid residue sequences derived from two or more Ad serotypes. Thus, unlike methods and constructs available prior to the advent of the present disclosure, this invention allows the greatest possible flexibility in the design and preparation of useful viral vectors and cell lines which support their construction and propagation -- all with a decreased risk of recombining with wild-type Ad to produce potentially-harmful recombinants.

In part, the present invention discloses a simpler, alternative means of reducing the recombination between viral and cellular sequences than those discussed in the art. One such means is to increase the size of the deletion in the recombinant virus and thereby reduce the extent of shared sequences between that virus and any Ad genes present in a packaging cell line e.g., the Ad5 genes in 293 cells, or the various Ad genes in the novel cell lines of the present invention.

Deletions of all or portions of structural genes of the adenovirus have been considered undesirable because of the anticipated deleterious effects such deletions would have on viral reproduction and packaging. Indeed, the use of "helper" viruses or plasmids has often been recommended when using Ad-derived vectors containing large deletions in structural protein sequences precisely for this reason.

Contrary to what has been suggested in the art, however, this invention discloses and claims the preparation, propagation and use of recombinant Ad-derived vectors having deletions of all or part of various gene sequences encoding Ad structural proteins, both as a means of reducing the risk of wild-type adenovirus contamination in virus preparations and as a means of allowing foreign DNA to be packaged in such vectors for a variety of diagnostic and therapeutic applications.

Thus, in one embodiment of the present invention, a packaging cell line wherein DNA sequences encoding one or more adenovirus regulatory polypeptides and DNA sequences encoding one or more adenovirus structural polypeptides have been stably integrated into the cellular genome is disclosed.

Thus, in a further embodiment of the present invention, a packaging cell line expressing one or more adenovirus structural proteins, polypeptides, or fragments thereof, wherein said structural protein is selected from the group consisting of:

- a. penton base;
- b. hexon;
- c. fiber;
- d. polypeptide IIIa;
- e. polypeptide V;
- f. polypeptide VI;

- g. polypeptide VII;
- h. polypeptide VIII; and
- i. biologically active fragments thereof is disclosed.

In one variation, the sequences are constitutively expressed; in another, one or more sequences is under the control of a regulatable promoter. In a preferred embodiment expression is constitutive. In various preferred embodiments, the polypeptides expressed by the DNA sequences are biologically active.

In a further and preferred embodiment the packaging cell line of the present invention supports the production of a viral vector. In a preferred embodiment the viral vector is a therapeutic vector.

In one aspect of the present invention, each DNA sequence is introduced into the genome of the within-disclosed cell lines via a separate complementing plasmid. In other embodiments, two or more DNA sequences were introduced into the genome via a single complementing plasmid. In one variation, the complementing plasmid comprises a DNA sequence encoding adenovirus fiber protein, polypeptide or fragment thereof. An example of a useful complementing plasmid according to the present invention is a plasmid having the characteristics of pCLF (for deposit details, see Example 3)

In another aspect of the present invention, the complementing plasmid used to transform a cell line of the present invention further comprises a DNA sequence encoding an adenovirus regulatory protein, polypeptide or fragment thereof. In one variation, the regulatory protein is selected from the group consisting of E1A, E1B, E2A, E2B, E3, E4 and L4 (also referred to as "the 100K protein"); an exemplary complementing plasmid has the characteristics of is pE4/Hygro?? (for deposit details, see Example 3). In another aspect, the complementing plasmid used to transform a cell line of the present invention further comprises a DNA sequence encoding two or more of the above mentioned adenovirus regulatory proteins, polypeptides or fragments thereof.

In one variation, the two or more regulatory proteins, polypeptides or fragments thereof are selected from the group consisting of E1A, E1B, E2A, E2B, E3, E4 and L4 (also referred to as "the 100K protein"). In another variation, the structural protein is selected from the group consisting of penton base; hexon; fiber; polypeptide IIIa; polypeptide V; polypeptide VI; polypeptide VII; polypeptide VIII; and biologically active fragments thereof.

In one variation of the present invention, a packaging cell line expresses fiber protein. In one embodiment, the fiber protein has been modified to include a non-native amino acid residue sequence which targets a specific receptor, but which does not disrupt trimer formation or transport of fiber into the nucleus. In another variation, the non-native amino acid residue sequence alters the binding specificity of the fiber for a targeted cell type. In still another embodiment, the structural protein is fiber comprising amino acid residue sequences from more than one adenovirus serotype. As disclosed herein, the nucleotide sequences encoding fiber protein or polypeptide need not be modified solely at one or both termini; fiber protein -- and indeed, any of the adenovirus structural proteins, as taught herein -- may be modified "internally" as well as at the termini.

The present invention also discloses a packaging cell line wherein the viral vector produced in that cell line comprises a nucleic acid sequence having a deletion or mutation of a DNA sequence encoding an adenovirus structural protein, polypeptide, or fragment thereof. In one variation, the viral vector further comprises a nucleic acid sequence having a deletion or mutation of the DNA sequences encoding regulatory polypeptides E1A and E1B. In another variation, the viral vector further comprises a nucleic acid sequence having a deletion or mutation of a DNA sequence encoding one or more of the following regulatory proteins or polypeptides: E2A, E2B, E3, E4, L4, or fragments thereof.

Yet another variation discloses that a foreign DNA sequence encoding one or more foreign proteins, polypeptides or fragments thereof has been inserted in place of any of the deletions in the therapeutic viral vector. In one embodiment, the foreign DNA encodes a tumor-suppressor protein or a biologically active fragment thereof. In another embodiment, the foreign DNA encodes a suicide protein or a biologically active fragment thereof. As before, cell lines as described herein may be prokaryotic or eucaryotic in origin, with mammalian cell lines often being preferred. Epithelial and non-epithelial cell lines are useful in the aforementioned variations; some particularly useful cell lines include 293, A549, W162, HeLa, Vero, 211, and 211A cell lines.

The invention further contemplates that the aforementioned cell lines support the production of viral vectors including foreign DNA sequences encoding one or more foreign proteins, polypeptides or fragments thereof has been inserted in place of any structural and/or

regulatory proteins (or portions thereof) that have been deleted. Thus, in one embodiment, the foreign DNA encodes a tumor-suppressor protein; a suicide protein; a cystic fibrosis transmembrane conductance regulator (CFTR) protein; or a biologically active fragment of any of them.

Any of the within-disclosed cell lines may have a DNA sequence encoding all or part of a fiber protein -- including modified or chimeric proteins -- stably integrated into the genome. thus, in one variation, the fiber protein has been modified to include a non-native amino acid residue sequence which targets a specific receptor, but which does not disrupt trimer formation or transport of fiber into the nucleus. In one variation, the non-native amino acid residue sequence is coupled to the carboxyl terminus of the fiber. In yet another, the non-native amino acid residue sequence further includes a linker sequence. Alternatively, the fiber protein further comprises a ligand coupled to the linker. A suitable ligand may be selected from the group consisting of ligands that specifically bind to a cell surface receptor and ligands that can be used to couple other proteins or nucleic acid molecules. In one variation, the ligand is selected from the group consisting of ligands that specifically bind to a cell surface receptor and ligands that can be used to couple other proteins or nucleic acid molecules.

In yet another embodiment, the non-native amino acid residue sequence is incorporated into the fiber amino acid residue sequence at a location other than one of the fiber termini. Alternatively, the non-native amino acid residue sequence alters the binding specificity of the fiber for a targeted cell type. In other embodiments, the linker sequence alters the binding specificity of the fiber for a targeted cell type. The expressed fiber may, in various embodiments, bind to a specific targeted cell type not usually targeted by adenovirus and/or may comprise amino acid residue sequences from more than one adenovirus serotype.

In various aspects of the present invention, a packaging cell line of the present invention is derived from a prokaryotic cell line; in another, it is derived from a eucaryotic cell line. While various embodiments suggest the use of mammalian cells, and more particularly, epithelial cell lines, a variety of other, non-epithelial cell lines are used in various embodiments. Thus, while various embodiments disclose the use of a cell line selected from the group consisting of 293, A549, W162, HeLa, Vero, 211, and 211A cell lines, it is understood that various other cell lines are likewise contemplated for use as disclosed herein.

The invention further discloses a wide variety of nucleic acid sequences and viral vectors. Thus, in one embodiment, the invention discloses a nucleic acid sequence encoding any one of the aforementioned adenovirus fiber proteins, polypeptides or fragments thereof -- including, without limitation, those that include deletions or other mutations; those that are chimeric; and those that have linkers, foreign amino acid residues, or other molecules attached for various purposes as disclosed herein. Nucleic acid sequences encoding various other adenovirus structural and/or regulatory proteins or polypeptides are also within the scope of the present invention.

A wide variety of therapeutic viral vectors are also embodiments of the present invention. In one embodiment, a therapeutic viral vector is disclosed which lacks a DNA sequence encoding fiber protein, or a portion thereof. In another variation, a therapeutic viral vector may further or alternatively comprise deletion of a DNA sequence encoding one or more regulatory proteins, polypeptides, or fragments thereof. In various embodiments, foreign DNA sequences are inserted in place of the DNA sequence encoding fiber protein in the viral vectors of the present invention. In other embodiments, the therapeutic viral vectors further comprise foreign DNA sequences inserted in place of the DNA sequences encoding one or more regulatory proteins, polypeptides, or fragments thereof, and/or one or more structural proteins, polypeptides, or fragments thereof.

The present invention further discloses a number of viral vectors. In one variation, a viral vector comprises a deletion or mutation of a DNA sequence encoding an adenovirus structural protein, polypeptide, or fragment thereof. A vector may further comprise deletion or mutation of the DNA sequences encoding regulatory polypeptides E1A and E1B; and it may still further comprise deletion or mutation of the DNA sequence encoding one or more of the following regulatory proteins or polypeptides: E2A, E2B, E3, E4, L4, or fragments thereof. In another variation, in a viral vector of the present invention, the structural protein comprises fiber. Any combination of the foregoing is also contemplated by the present invention. The viral vectors of the present invention are suitable for the preparation of pharmaceutical compositions comprising any of the therapeutic viral vectors disclosed herein -- including combinations thereof -- are also disclosed herein. A further use of the viral vectors of the present invention is for targeting specific cells in a cell population comprising different cell types.

The invention further discloses complementing plasmids and methods of making same. In one embodiment, a complementing plasmid comprises a promoter nucleotide sequence operatively linked to a nucleotide sequence encoding an adenovirus structural polypeptide. In one variation, the complementing plasmid comprises pCLF. In another variation, a complementing plasmid further comprises a nucleotide sequence encoding a first adenovirus regulatory polypeptide, a nucleotide sequence encoding a second regulatory polypeptide, a nucleotide sequence encoding a third regulatory polypeptide; or any combination of the foregoing. In still another embodiment, the adenovirus structural polypeptide is selected from the group consisting of penton base; hexon; fiber; polypeptide IIIa; polypeptide V; polypeptide VI; polypeptide VII; polypeptide VIII; and biologically active fragments thereof.

The present invention also discloses a complementing plasmid comprising a promoter nucleotide sequence operatively linked to a nucleotide sequence encoding an adenovirus structural protein, polypeptide or fragment thereof and a nucleotide sequence encoding an adenovirus regulatory protein, polypeptide or fragment thereof. In one variation, the early region polypeptide is E4; in another, the plasmid comprises pE4/Hygro. In still another variation, the early region polypeptides are E1 and E4. Complementing plasmids further comprising a nucleotide sequence encoding an adenovirus structural protein, polypeptide or fragment thereof are also contemplated, as are plasmids wherein the promoter nucleotide sequence is selected from the group consisting of MMTV, CMV and E4 promoter nucleotide sequences.

Viral vectors are also disclosed which comprise nucleotide sequences encoding a packaging signal and a foreign protein or polypeptide, wherein the nucleotide sequence encoding an adenovirus structural protein has been deleted. In one variation, the nucleotide sequence encoding the foreign protein or polypeptide is a DNA molecule up to about 3 kb in length; in another, the nucleotide sequence encoding the foreign protein or polypeptide is a DNA molecule up to about 9.5 kb in length; in still another, the nucleotide sequence encoding the foreign protein or polypeptide is a DNA molecule up to about 12.5 kb in length. Nucleotide sequences of intermediate lengths are also contemplated by the present invention, as are sequences in excess of 12.5 kb.

The invention also discloses viral vectors wherein the sequence encoding a foreign protein or polypeptide is a sequence encoding an anti-tumor agent, a tumor suppressor protein,

a suicide protein, or a fragment or functional equivalent thereof. In one variation, nucleotide sequences encoding one or more regulatory proteins have also been deleted from the vector. In another, the regulatory proteins are selected from the group consisting of E1A, E1B, E2A, E2B, E3, E4, and L4 (100K protein).

In various embodiments, the adenovirus is a Group C adenovirus selected from serotypes 1, 2, 5, or 6; in other embodiments, adenovirus selected from other serotypes are useful as disclosed herein. The invention also discloses useful vaccines comprising a viral vector according to any of the foregoing specifications, and a pharmaceutically acceptable carrier or excipient.

Various useful compositions are also disclosed herein. One embodiment discloses a composition useful in the preparation of recombinant adenovirus viral vectors comprising a cell containing a delivery plasmid comprising an adenovirus genome lacking a nucleotide sequence encoding fiber. In one variation, the cell further comprises a complementing plasmid containing a nucleotide sequence encoding fiber, the plasmid being stably integrated into the cellular genome of the cell. In another variation, the delivery plasmid further comprises a nucleotide sequence encoding a foreign polypeptide. In one variation, the delivery plasmid is pDV44, p E1B gal, or p E1sp1B.

In another embodiment, the polypeptide is a therapeutic molecule. In yet another, the polypeptide is a therapeutic molecule. Another variation provides that the delivery plasmid further comprises a nucleotide sequence encoding a foreign polypeptide.

Compositions useful in the preparation of recombinant adenovirus viral vectors are also disclosed. In one embodiment, a composition comprises a cell containing a first delivery plasmid comprising an adenovirus genome lacking a nucleotide sequence encoding fiber and incapable of directing the packaging of new viral particles in the absence of a second delivery plasmid; and a second delivery plasmid comprising an adenoviral genome capable of directing the packaging of new viral particles in the presence of the first delivery plasmid.

In another variation, the first and second delivery plasmids interact within the cell to produce a therapeutic viral vector. In yet another, the cell further comprises a complementing plasmid containing a nucleotide sequence encoding fiber, the plasmid being stably integrated into the cellular genome of the cell. In still another, the first or second delivery plasmid

further comprises a nucleotide sequence encoding a foreign polypeptide. In various embodiments, the polypeptide is a therapeutic molecule.

Another embodiment discloses a composition as before, wherein the first delivery plasmid lacks adenovirus packaging signal sequences. In another aspect, the second delivery plasmid contains a LacZ reporter construct. Another variation discloses that the second delivery plasmid further lacks a nucleotide sequence encoding an adenovirus regulatory protein. In one variation, the regulatory protein is E1. In one embodiment of the above-noted compositions, the complementing plasmid has the characteristics of pCLF.

In another embodiment, a composition is disclosed wherein the first delivery plasmid lacks a nucleotide sequence encoding an adenovirus structural protein and the second delivery plasmid lacks a nucleotide sequence encoding adenovirus E1 protein. In another, the first delivery plasmid lacks a nucleotide sequence encoding adenovirus E4 protein and the second delivery plasmid lacks a nucleotide sequence encoding adenovirus E1 protein. In still another, the cell contains at least one complementing plasmid encoding an adenoviral regulatory protein and a structural protein.

In alternative embodiments, the regulatory protein is E4 and the structural protein is fiber; or the regulatory protein is E1 and the structural protein is fiber. In still another embodiment, the adenoviral regulatory protein and the structural protein are encoded by separate complementing plasmids.

Another variation discloses a composition wherein the cell is selected from the group consisting of 293, A549, W162, HeLa, Vero, 211, and 211A. In another embodiment, the delivery plasmid is DV1, or p E1B gal, or p E1sp1B.

Various methods of making and using the vectors, plasmids, cell lines and other compositions and constructs of the present invention are also disclosed herein. The following methods are considered exemplary and not limiting.

Thus, in one variation, the invention discloses a method of constructing therapeutic viral vectors, comprising introducing a delivery plasmid into an Ad fiber-expressing complementing cell line, wherein the DNA sequence encoding Ad fiber protein has been deleted from the delivery plasmid. In one variation, the delivery plasmid further includes a DNA sequence encoding a foreign protein, polypeptide, or fragment thereof. In other embodiments, the delivery plasmid is DV1, p E1B gal, p E1sp1B, or similar constructs.

The invention also discloses methods of transforming a pathologic hyperproliferative mammalian cell comprising contacting the cell with any of the vectors described herein. In another embodiment, methods of infecting a mammalian target cell with a viral vector containing a preselected foreign nucleotide sequence are disclosed. One such variation comprises the following steps: (a) infecting the target cell with a viral vector of the present invention, the viral vector carrying a preselected foreign nucleotide sequence; and (b) expressing the foreign nucleotide sequence in the targeted cell.

The invention also encompasses mammalian target cells infected with a preselected foreign nucleotide sequence produced by the methods disclosed herein. In one variation, the target cells are selected from the group consisting of replicating, slow-replicating and non-replicating human cells.

Methods of treating an acquired or hereditary disease are also disclosed. One method comprises (a) administering a pharmaceutically acceptable dose of a viral vector to a target cell, wherein the vector comprises a preselected therapeutic nucleotide sequence; and (b) expressing the therapeutic sequence in the target cell for a time period sufficient to ameliorate the acquired or hereditary disease in the cell. Method of gene therapy comprising administering to a subject an effective amount of a therapeutic viral vector produced by a packaging cell line of the present invention are also disclosed.

Also contemplated by the present invention are various methods of inhibiting the proliferation of a tumor in a subject comprising administering an effective amount of a therapeutic viral vector of the present invention under suitable conditions to the subject. IN one variation, the gene encodes an anti-tumor agent. In another variation, the agent is a tumor-suppressor gene. In still another embodiment, the agent is a suicide gene or a functional equivalent thereof. In another variation, the vector is administered via intra-tumoral injection.

The invention also discloses systems or kits for use in any of the aforementioned methods. The systems or kits may contain any appropriate combination of the within-described vectors, plasmids, cell lines, and additional therapeutic agents as disclosed. Preferably, each such kit or system includes a quantity of the appropriate therapeutic substance or sequence sufficient for at least one administration, and instructions for administration and use. Thus, one system further comprises an effective amount of a therapeutic agent which enhances the therapeutic effect of the therapeutic viral vector-containing composition.

Another variation discloses that the composition and the therapeutic agent are each included in a separate receptacle or container.

It will also be appreciated that any combination of the preceding elements may also be efficacious as described herein, and that all related methods are also within the scope of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the entire adenoviral E4 transcriptional unit with the open reading frames (ORF) indicated by blocked segments along with the promoter and terminator sequences. The location of primers for amplifying specific portions of E4 are also indicated as further described in Example 1A.

Figure 2 is a schematic map of plasmid pE4/Hygro as further described in Example 1B.

Figure 3 is a schematic map of plasmid pCDNA3/Fiber as further described in Example 1B.

Figure 4 is a schematic map of plasmid pCLF as further described in Example 1B.

Figure 5 is a photograph of a Southern blot showing the presence of intact adenovirus E4 3.1 kilobase (kb) insert in the 211 cell line as further described in Example 1C.

Figure 6 is a photograph of a Western blot showing labeled fiber protein detected under native and denaturing electrophoresis conditions as described in Example 1C. The 293 cells lack fiber while the sublines 211A, 211B and 211R contain fiber protein detectable in functional trimerized form and denatured monomeric form.

Figure 7 is a schematic map of plasmid pDEX/E1 as further described in Example 1D.

Figure 8 is a schematic map of plasmid pE1/Fiber as further described in Example 1F1.

Figure 9 is a schematic map of plasmid pE4/Fiber as further described in Example 1F2).

Figure 10 is a schematic illustration of linearized ϕ E1B gal delivery plasmids for use in cotransfection and recombination to form a recombinant adenoviral vector having multiple adenoviral gene deletions. The plasmids and recombination event are more fully described in Example 2A.

Figure 11 is a schematic of plasmid p11.3 as further described in Example 2A used in the construction of pDV44 delivery plasmid with plasmid p8.2.

Figure 12 is a schematic of plasmid p8.2 as further described in Example 2A used in the construction of pDV44 delivery plasmid with plasmid p11.3.

Figure 13. Trimeric structure of the recombinant fiber: 293, 211A, 211B, or 211R cells as indicated were metabolically labeled with [³⁵S)methionine, soluble protein extracts prepared, and fiber was immunoprecipitated. A portion of the precipitated protein was electrophoresed on an 8% SDS-PAGE gel under either semi-native or denaturing conditions. The positions of trimeric (T) and monomeric (M) fiber are indicated. As a control for electrophoretic conditions, recombinant Ad2 fiber produced in baculovirus-infected cells was run under identical conditions and stained with Coomassie blue.

Fig. 14. Complementation of a fiber mutant adenovirus by fiber-producing cells: The cell lines indicated (2×10^6 cells per sample) were infected with the temperature-sensitive fiber mutant adenovirus H5ts142 at 10 PFU/cell and incubated at either the permissive (32.5 °C, stippled bars) or the restrictive (39.5 °C, solid bars) temperature. 48 hours post-infection, virus was isolated by freeze-thaw lysis and yields determined by fluorescent focus assay on SW480 cells. Each value represents the mean of duplicate samples, and the data shown is representative of multiple experiments.

Fig. 15. Incorporation of the recombinant Ad5 fiber into Ad3 particles: A) Alignment of the N-terminal (penton base-binding) domains of fiber proteins from several different adenovirus serotypes. B) Type 3 adenovirus was propagated in 293, 211B, or 211R cells as indicated and purified by two sequential CsCl centrifugations. 10 mg of the purified viral particles was then electrophoresed under denaturing conditions and transferred to a PVDF membrane. Ad5 fiber was detected with a polyclonal rabbit antibody raised against recombinant Ad2 fiber. As a positive control for detection, 400 ng of wild-type Ad2 was run in the lane marked 'Ad2'. Under these conditions, the mobilities of the Ad2 and Ad5 fibers are indistinguishable and the antibody reacts with both proteins.

Fig. 16. Nuclear localization of the recombinant fiber protein in three packaging cell lines: Cells were grown on 8-well chamber slides, stained with a rabbit anti-fiber polyclonal antibody and visualized

with a FITC-conjugated goat anti-rabbit antibody. A) line 211A. B) Line 211B C) Line 211R D) 293 cells (negative control). E) 293 cells infected with Ad.RSVbgal at 1 pfu/cell and stained 24 hour post-infection (positive control). F) Infected cells prepared as in (E) but stained without the primary antibody.

DETAILED DESCRIPTION To reduce the frequency of contamination with wild-type adenovirus, it is considered desirable to improve either the viral vector or the cell line to reduce the probability of recombination. For example, an adenovirus from a group with less homology to the group C viruses may be used to engineer recombinant viruses with little propensity for recombination with the Ad5 sequence in 293 cells. Similarly, an epithelial cell line -- e.g. the cell line known as 293 -- may be used or further modified according to within-disclosed methods which stably expresses adenovirus proteins or polypeptides from Ad3 and/or proteins or polypeptides from another non-group-C or group C serotype; such a cell line would be useful to support adenovirus-derived viral vectors bearing deletions of regulatory and/or structural genes, irrespective of the serotype from which such a vector was derived.

It is also contemplated that the constructs and methods of the present invention will support the design and engineering of chimeric viral vectors which express amino acid residue sequences derived from two or more Ad serotypes. Thus, unlike methods and constructs available prior to the advent of the present disclosure, this invention allows the greatest possible flexibility in the design and preparation of useful viral vectors and cell lines which support their construction and propagation -- all with a decreased risk of recombining with wild-type Ad to produce potentially-harmful recombinants.

In part, the present invention discloses a simpler, alternative means of reducing the recombination between viral and cellular sequences than those discussed in the art. One such means is to increase the size of the deletion in the recombinant virus and thereby reduce the

extent of shared sequences between that virus and any Ad genes present in a packaging cell line

— e.g., the Ad5 genes in 293 cells, or the various Ad genes in the novel cell lines of the present invention.

By the term "substantially homologous" is meant having at least 80%, preferably at least 90%, most preferably at least 95% homology therewith.

The amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH₂ refers to the free amino group present at the amino terminus of a polypeptide.

DNA Homolog: A nucleic acid having a preselected conserved nucleotide sequence and a sequence encoding a preferred polypeptide according to the present invention.

Foreign Gene: This term is used to identify a DNA molecule not present in the exact orientation and position as the counterpart DNA molecule found in wild-type adenovirus. It may also refer to a DNA molecule from another Ad serotype or from an entirely different species -- e.g. a human DNA sequence.

Penton: The terms "penton" or "penton complex" are preferentially used herein to designate a complex of penton base and fiber. The term "penton" may also be used to indicate penton base, as well as penton complex. The meaning of the term "penton" alone should be clear from the context within which it is used.

Polypeptide and Peptide: These terms are used interchangeably herein to designate a series of no more than about 50 amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues.

Receptor: Receptor is a term used herein to indicate a biologically active molecule that specifically binds to (or with) other molecules. The term "receptor protein" may be used to more specifically indicate the proteinaceous nature of a specific receptor.

Transgene or Therapeutic Nucleotide Sequence: As described and claimed herein, such a sequence includes DNA and RNA sequences encoding an RNA or polypeptide. Such sequences may be "native" or naturally-derived sequences; they may also be "non-native" or "foreign" sequences which are naturally- or recombinantly-derived. The term "transgene," which may be used interchangeably herein with the term "therapeutic nucleotide sequence," is often used to describe a heterologous or foreign (exogenous) gene that is carried by a viral vector and transduced into a host cell.

Therefore, therapeutic nucleotide sequences include antisense sequences or nucleotide sequences which may be transcribed into antisense sequences. Therapeutic nucleotide sequences (or transgenes) further comprise sequences which function to produce a desired effect in the cell or cell nucleus into which said therapeutic sequences are delivered. For example, a therapeutic nucleotide sequence may encode a functional protein intended for delivery into a cell which is unable to produce that functional protein.

Expression or Delivery Vector: Any plasmid or virus into which a foreign DNA may be inserted for expression in a suitable host cell -- i.e., the protein or polypeptide encoded by the DNA is synthesized in the host cell's system. Vectors capable of directing the expression of DNA segments (genes) encoding one or more proteins are referred to herein as "expression vectors". Also included are vectors which allow cloning of cDNA (complementary DNA) from mRNAs produced using reverse transcriptase.

Adenoviral Vector or Ad-Derived Vector: Any adenovirus-derived plasmid or virus into which a foreign DNA may be inserted or expressed. This term may also be used interchangeably with "viral vector" This "type" of vector may be utilized to carry nucleotide

sequences encoding therapeutic proteins or polypeptides to specific cells or cell types in a subject in need of treatment, as described further hereinbelow.

Complementing Plasmid: This term is generally used herein to describe plasmid vectors used to deliver particular nucleotide sequences into a packaging cell line, with the intent of having said sequences stably integrate into the cellular genome.

Delivery Plasmid: This term is generally used herein to describe a plasmid vector that carries or delivers nucleotide sequences in or into a cell line (e.g., a packaging cell line) for the purpose of propagating therapeutic viral vectors of the present invention.

The adenovirus (Ad) particle is relatively complex and may be resolved into various substructures. The outer shell is strikingly icosahedral in shape and, at first glance, appears to have a triangulation number of 25. The structures at the fivefold positions ("pentons") are different from the rest ("hexons"), however, and the hexons are chemically trimers rather than hexamers. Thus, the structure really does not correspond to a simple sub-triangulated icosahedral design. (See, e.g., Fields, et al., Virology, Vol. I, Raven Press, NY, pp. 54-56 (1990).)

Fiber plays a crucial role in adenovirus infection by attaching the virus to a specific receptor on the cell surface. The fiber consists of three domains: an N-terminal tail that interacts with penton base; a shaft composed of 22 repeats of a 15-amino-acid segment that forms α -sheet and β -bends; and a knob at the C-terminus that contains the type-specific antigen and is responsible for binding to the cell surface receptor. The fiber protein is also responsible for transport of viral nucleic acids into the nucleus. The gene encoding the fiber protein from Ad2 has been expressed in human cells and has been shown to be correctly assembled into trimers, glycosylated and transported to the nucleus. (See, e.g., Hong and Engler, Virology 185: 758-761 (1991).) Thus, alteration of gene delivery mediated by recombinant adenovirus vectors to specific cell types has great utility for a variety of gene therapy applications and is thus one of the objects of the present invention.

Hexon and penton capsomeres are the major components on the surface of the virion. Their constituent polypeptides, nos. II, III and IV, contain tyrosine residues that are exposed on the surface of the virion and can be labeled -- e.g., by iodination of intact particles.

The fiber is an elongated protein which exists as a trimer of three identical polypeptides (polypeptide IV) of 582 amino acids in length. The N-terminus of the fiber mediates binding to the penton base to form what is generally called the penton capsomere. The C-terminus of the fiber is involved in initial binding of the virus to cellular receptors.

The 35,000+ base pair (bp) genome of adenovirus type 2 has been sequenced and the predicted amino acid sequences of the major coat proteins (hexon, fiber and penton base) have been described. (See, e.g., Neumann et al., Gene 69: 153-157 (1988); Herisse et al., Nuc. Acids Res. 9: 4023-4041 (1981); Roberts et al., J. Biol. Chem. 259: 13968-13975 (1984); Kinloch et al., J. Biol. Chem. 259: 6431-6436 (1984); and Chroboczek et al., Virol. 161: 549-554 (1987).)

The sequence of Ad5 DNA was completed more recently; its sequence includes a total of 35,935 bp. Portions of many other adenovirus genomes have also been sequenced. It is presently understood that the upper packaging limit for adenovirus virions is about 105% of the wild-type genome length. (See, e.g., Bett, et al., J. Virol. 67(10): 5911-21 (1993).) Thus, for Ad2 and Ad5, this would be an upper packaging limit of about 38kb of DNA.

Adenovirus DNA also includes inverted terminal repeat sequences (ITRs) ranging in size from about 100 to 150 bp, depending on the serotype. The inverted repeats enable single strands of viral DNA to circularize by base-pairing of their terminal sequences, and the resulting base-paired "panhandle" structures are thought to be important for replication of the viral DNA.

For efficient packaging, the ITRs and the packaging signal (a few hundred bp in length) appear to comprise the "minimum requirement." Helper-dependent vectors lacking all viral ORFs but including these essential *cis* elements (the ITRs and contiguous packaging sequence) have been constructed, but the virions package less efficiently than the helper and package as multimers part of the time, which suggests that the virus may "want" to package a fuller DNA complement (see, e.g., Fisher, et al., Virology 217: 11-22 (1996)).

While some prefer to use replication-defective Ad viral vectors for fear that replication-competent vectors raise safety issues, the viral vectors of the present invention may retain their ability to express the genome packaged within -- i.e., they may retain their "infectivity" -- they do not act as infectious agents to the extent that they cause disease in the subjects to whom they are administered for therapeutic purposes.

It is to be appreciated that the viral vectors of the present invention have several distinct advantages over adenoviral and Ad-derived vectors described in the art. For example, recombination of such vectors is rare; there are no known associations of human malignancies with adenoviral infections despite common human infection with adenoviruses; the genome may be manipulated to accommodate foreign genes of a fairly substantial size; and host proliferation is not required for expression of adenoviral proteins.

An extension of this invention is that the Ad-derived viral vectors disclosed herein may be used to target and deliver genes into specific cells by incorporating the attachment sequence for other receptors (such as CD4) onto the fiber protein by recombinant DNA techniques, thus producing a chimeric molecule. This should result in the ability to target and deliver genes into a wide range of cell types with the advantage of evading recognition by the host's immune system. The within-disclosed delivery systems thus provide for increased flexibility in gene design to enable stable integration into proliferating and nonproliferating cell types.

For example, published International App. No. WO95/26412 and Krasnykh, et al. (J. Virol. 70: 6839-46 (1996)), the disclosures of which are incorporated by reference herein, describe modifications that may be made to the adenovirus fiber protein. Such modifications are useful in altering the targeting mechanism and specificity of adenovirus and could readily be utilized in conjunction with the constructs of the present invention to target the novel viral vectors disclosed herein to different receptors and different cells. Moreover, modifications to fiber protein which alter its tropism may permit greater control over the localization of viral vectors in therapeutic applications.

Similarly, incorporation of various structural proteins into cell lines of the present invention, whether or not those proteins are modified, is also contemplated by the present invention. Thus, for example, modified penton base polypeptides such as those described in Wickham, et al. (J. Virol. 70: 6831-8 (1996)) may have therapeutic utility when used according to the within-disclosed methods.

C. Packaging Cell Lines

The first generation of recombinant adenoviral vectors currently available tend to have a deletion in the first viral early gene region which is generally referred to as E1, which comprises the E1a and E1b regions. (These regions typically span genetic map units 1.30 to 9.24.) Figure 3 in chapter 67 of Fields Virology, 3d Ed. (Fields et al. (eds.), Lippincott-Raven Publ., Philadelphia, (1996), p. 2116) illustrates a transcription and translation map of adenovirus type 2 (Ad2) that is a helpful example.

According to various published reports, deletion of the viral E1 region renders the recombinant adenovirus defective for replication and incapable of producing infectious viral particles in the subsequently-infected target cells. Thus, the ability to generate E1-deleted adenovirus is often based on the availability of the human embryonic kidney packaging cell line called 293. This cell line contains the E1 region of adenovirus, which provides E1 gene region products to "support" the growth of E1-deleted virus in the cell line (see, e.g., Graham et al., J. Gen. Virol. 36: 59-71 (1977)).

Nevertheless, the inherent problems with current first-generation recombinant adenoviruses have raised increasing concerns about their use in patients. For example, several recent studies have shown that E1-deleted adenoviruses are not completely replication-incompetent (see Rich, Hum. Gene. Ther. 4: 461-476 (1993); Engelhardt, et al., Nature Genet. 4: 27-34 (1993)).

Three general limitations are associated with the adenoviral vector technology. First, infection both *in vivo* and *in vitro* with the adenoviral vector at high multiplicity of infection ("MOI") has resulted in cytotoxicity to the target cells, due to the accumulation of penton protein, which is itself toxic to mammalian cells (Kay, Cell Biochem. 17E: 207 (1993)). Second, host immune responses against adenoviral late gene products, including penton protein, cause the inflammatory response and destruction of the infected tissue which received the vectors (Yang, et al., PNAS USA 92: 4407-4411 (1994)). Lastly, host immune responses and cytotoxic effects together prevent the long-term expression of transgenes and cause decreased levels of gene expression following subsequent administration of adenoviral vectors (Mittal, et al., Virus Res. 28: 67-90 (1993)).

The packaging cell lines disclosed herein support viral vectors with deletions of major portions of the viral genome, without the need for helper viruses.

D. Therapeutic Viral Vectors and Related Systems

1. Nucleic Acid Segments

A therapeutic viral vector or composition of the present invention comprises a nucleotide sequence encoding a protein or polypeptide molecule -- or a biologically active fragment thereof -- which may be used for therapeutic applications, as described herein. A therapeutic viral vector or composition may further comprise an enhancer element or a promoter located 5' to and controlling the expression of such a therapeutic nucleotide sequence or gene.

In general, promoters are DNA segments that contain a DNA sequence that controls the expression of a gene located 3' or downstream of the promoter. The promoter is the DNA sequence to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene, typically located 3' of the promoter. If more than one nucleic acid sequence encoding a particular polypeptide or protein is included in a therapeutic viral vector or nucleotide sequence, more than one promoter or enhancer element may be included, particularly if that would enhance efficiency of expression. For purposes of the present invention, regulatable (inducible) as well as constitutive promoters may be used, either on separate vectors or on the same vector.

A subject therapeutic nucleotide composition or vector consists of a nucleic acid molecule that comprises at least 2 different operatively linked DNA segments. The DNA can be manipulated and amplified by PCR as described herein and by using standard techniques, such as those described in Molecular Cloning: A Laboratory Manual, 2nd Ed., Sambrook et al., eds., Cold Spring Harbor, New York (1989). Typically, to produce a therapeutic viral vector of the present invention, the sequence encoding the selected therapeutic composition and the promoter or enhancer are operatively linked to a DNA molecule capable of autonomous replication in a cell either *in vivo* or *in vitro*. By operatively linking the enhancer element or promoter and the nucleotide sequence encoding the therapeutic nucleotide composition to the vector, the attached segments are replicated along with the vector sequences.

Thus, a recombinant DNA molecule (rDNA) of the present invention is a hybrid DNA molecule comprising at least 2 nucleotide sequences not normally found together in nature. In various preferred embodiments, one of the sequences is a sequence encoding an Ad-derived polypeptide, protein, or fragment thereof. Stated another way, a therapeutic nucleotide sequence of the present invention is one that encodes an expressible protein, polypeptide or fragment thereof, and it may further include an active constitutive or regulatable (e.g. inducible) promoter sequence.

A therapeutic viral vector or composition of the present invention is optimally from about 20 base pairs to about 40,000 base pairs in length. Preferably the nucleic acid molecule is from about 50 bp to about 38,000 bp in length. In various embodiments, the nucleic acid molecule is of sufficient length to encode one or more adenovirus proteins or functional polypeptide portions thereof. Since individual Ad polypeptides vary in length from about 19 amino acid residues to about 967 amino acid residues, corresponding nucleotide sequences will range from about 50 bp up to about 3000 bp, depending on the size and of individual polypeptide-encoding sequences that are "replaced" in the viral vectors by therapeutic nucleotide sequences of the present invention.

Various Ad proteins are comprised of more than one polypeptide sequence. Thus, deletion of the corresponding genes from an Ad vector as taught herein will thus allow the vector to accommodate even larger "foreign" DNA segments. Thus, if the sequences encoding one or more adenovirus polypeptides or proteins are supplanted by a recombinant nucleotide sequence of the present invention, the length of the recombinant sequence can conceivably extend nearly to the packaging limit of the relevant adenovirus-derived vector.

In view of the fact that preferred embodiments disclosed herein are helper-independent Ad-derived vectors, the entire wild-type Ad genome cannot be completely supplanted by recombinant nucleic acid molecules without transforming such a vector into a vector requiring "help" of some kind. However, the Ad-derived vectors of the present invention do not depend on a helper virus; instead, the vectors of the present invention are propagated in cell lines stably expressing proteins or polypeptides that have been removed from said vectors to allow the addition of "foreign" DNA into the vectors. In various disclosed embodiments, specific early region and structural polypeptides are deleted from the vectors of the present invention, thereby enabling the vectors to accommodate recombinant nucleic acid sequences (or

cassettes) of various lengths. For example, Ad-derived vectors of the present invention may easily include 12 kb or more of foreign (or "therapeutic") DNA sequences.

The therapeutic (or foreign) nucleotide sequence can be a gene or gene fragment that encodes a protein or polypeptide -- or a biologically active fragment thereof -- that provides a desired therapeutic effect such as replacement of alpha 1-antitrypsin or cystic fibrosis transmembrane conductance regulator protein (CFTR) and the like. (See, e.g., Crystal, et al., Nature Genetics 8: 42-51 (1994); Zabner, et al., Cell 75: 207-216 (1993); Knowles, et al., NEJM 333(13): 823-831 (1995); and Rosenfeld, et al., Cell 68: 143-155 (1992), the disclosures of which are incorporated by reference herein.)

An Ad-derived vector of the present invention may also comprise a nucleotide sequence encoding a protein, polypeptide or fragment thereof that is effective in regulating the cell cycle -- such as p53, Rb, or mitosin -- or which is effective in inducing cell death, such as thymidine kinase. (See, e.g., published International App. No. WO 95/11984, the disclosures of which are incorporated by reference herein.) It is further contemplated that a therapeutic protein or polypeptide expressed by a therapeutic viral vector of the present invention may be used in conjunction with another therapeutic agent when appropriate -- e.g., a thymidine kinase metabolite may be used in conjunction with the gene encoding thymidine kinase and its gene product -- in order to be even more effective.

Alternatively, a therapeutic viral vector can include a DNA or RNA oligonucleotide sequence that exhibits enzymatic therapeutic activity without needing to be translated into a polypeptide product before exerting a therapeutic effect. Examples of the latter include antisense oligonucleotides that will inhibit the transcription of deleterious genes or ribozymes that act as site-specific ribonucleases for cleaving selected mutated gene sequences. In another variation, a therapeutic nucleotide sequence of the present invention may comprise a DNA construct capable of generating therapeutic nucleotide molecules, including ribozymes and antisense DNA, in high copy numbers in target cells, as described in published PCT application No. WO 92/06693 (the disclosure of which is incorporated herein by reference). Other preferred therapeutic nucleotide sequences according to the present invention are capable of delivering HIV antisense nucleotides to latently-infected T cells via CD4. Similarly, delivery of Epstein-Barr Virus (EBV) EBNA-1 antisense nucleotides to B cells via CR2 is capable of effecting therapeutic results.

As noted elsewhere herein, an Ad-derived vector of the present invention may also include a promoter sequence. Both constitutive and regulatable (often called "inducible") promoters are useful in constructs and methods of the present invention. For example, some useful regulatable promoters are those of the CREB-regulated gene family and include - and inhibin, - gonadotropin, cytochrome c, glucagon, and the like. (See, e.g., published International App. No. WO96/14061, the disclosures of which are incorporated by reference herein.)

A regulatable or inducible promoter may be described as a promoter wherein the rate of RNA polymerase binding and initiation is modulated by external stimuli. Such stimuli include various compounds or compositions, light, heat, stress, chemical energy sources, and the like. Inducible, suppressible and repressible promoters are considered regulatable promoters.

Regulatable promoters may also include tissue-specific promoters. Tissue-specific promoters direct the expression of the gene to which they are operably linked to a specific cell type. Tissue-specific promoters cause the gene located 3' of it to be expressed predominantly, if not exclusively, in the specific cells where the promoter expressed its endogenous gene. Typically, it appears that if a tissue-specific promoter expresses the gene located 3' of it at all, then it is expressed appropriately in the correct cell types (see, e.g., Palmiter et al., Ann. Rev. Genet. 20: 465-499 (1986)).

When a tissue-specific promoter controls the expression of a gene, that gene will be expressed in a small number of tissues or cell types rather than in substantially all tissues and cell types. Examples of tissue-specific promoters include the immunoglobulin promoter described by Brinster et al., Nature 306: 332-336 (1983) and Storb et al., Nature 310: 238-231 (1984); the elastase-I promoter described by Swift et al., Cell 38: 639-646 (1984); the globin promoter described by Townes et al., Mol. Cell. Biol. 5: 1977-1983 (1985), and Magram et al., Mol. Cell. Biol. 9: 4581-4584 (1989), the insulin promoter described by Bucchini et al., PNAS USA 83: 2511-2515 (1986) and Edwards et al., Cell 58: 161 (1989); the immunoglobulin promoter described by Ruscon et al., Nature 314: 330-334 (1985) and Grosschedl et al., Cell 38: 647-658 (1984); the alpha actin promoter described by Shani, Mol. Cell. Biol. 6: 2624-2631 (1986); the alpha crystalline promoter described by Overbeek et al., PNAS USA 82: 7815-7819 (1985); the prolactin promoter described by Crenshaw et al., Genes and Development 3: 959-972 (1989); the proopiomelanocortin promoter described by

Tremblay et al., PNAS USA 85: 8890-8894 (1988); the beta-thyroid stimulating hormone (BTSH) promoter described by Tatsumi et al., Nippon Rinsho 47: 2213-2220 (1989); the mouse mammary tumor virus (MMTV) promoter described by Muller et al., Cell 54: 105 (1988); the albumin promoter described by Palmiter et al., Ann. Rev. Genet. 20: 465-499 (1986); the keratin promoter described by Vassar et al., PNAS USA 86: 8565-8569 (1989); the osteonectin promoter described by McVey et al., J. Biol. Chem. 263: 11,111-11,116 (1988); the prostate-specific promoter described by Allison et al., Mol. Cell. Biol. 9: 2254-2257 (1989); the opsin promoter described by Nathans et al., PNAS USA 81: 4851-4855 (1984); the olfactory marker protein promoter described by Danciger et al., PNAS USA 86: 8565-8569 (1989); the neuron-specific enolase (NSE) promoter described by Forss-Peltier et al., J. Neurosci. Res. 16: 141-151 (1986); the L-7 promoter described by Sutcliffe, Trends in Genetics 3: 73-76 (1987) and the protamine 1 promoter described Peschon et al., Ann. New York Acad. Sci. 564: 186-197 (1989) and Braun et al., Genes and Development 3: 793-802 (1989). (The disclosures of all references cited are incorporated by reference herein.)

2. Compositions

In various alternative embodiments of the present invention, therapeutic sequences and compositions useful for practicing the therapeutic methods described herein are contemplated. Therapeutic compositions of the present invention may contain a physiologically tolerable carrier together with one or more therapeutic nucleotide sequences of this invention, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the composition is not immunogenic or otherwise able to cause undesirable side effects when administered to a subject for therapeutic purposes.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a subject -- e.g., a mammal -- without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

For example, the present invention comprises therapeutic compositions useful in the specific targeting of epithelial or non-epithelial cells as well as in delivering a therapeutic nucleotide sequence to those cells. Therapeutic compositions designed to preferentially target to epithelial cells may comprise an adenovirus-derived vector including a therapeutic

nucleotide sequence. As described herein, a number of adenovirus-derived moieties are useful in the presently-disclosed therapeutic compositions and methods.

While some of the Examples appearing below specifically recite fiber proteins, polypeptides, and fragments thereof, it is expressly provided herein that other structural and non-structural Ad proteins and polypeptides (e.g., regulatory proteins and polypeptides) may be used as components of the various disclosed vectors and cell lines. Moreover, chimeric molecules comprised of proteins, polypeptides, and/or fragments thereof which are derived from different Ad serotypes may be used in any of the within-disclosed methods, constructs and compositions. Similarly, recombinant DNA sequences of the present invention may be prepared using nucleic acid sequences derived from different Ad serotypes, in order to design useful constructs with broad applicability, as disclosed and claimed herein.

It should also be appreciated that, while the members of Group C adenovirus -- i.e., Ad serotypes 1, 2, 5, and 6 -- are specifically recited in various examples herein, the present invention is in no way limited to those serotypes alone. In view of the fact that the adenovirus serotypes are all closely-related in structure and functionality, therapeutic viral vectors, packaging cell lines, and plasmids of the present invention may be constructed from components of any and all Ad serotypes -- and the within-disclosed methods of making and using the various constructs and cell lines of the present invention apply to all of said serotypes.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art. Typically such compositions are prepared as injectables -- either as liquid solutions or suspensions -- however, solid forms suitable for solution or suspension in liquid prior to use can also be prepared. A preparation can also be emulsified, or formulated into suppositories, ointments, creams, dermal patches, or the like, depending on the desired route of administration.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof, including vegetable oils, propylene glycol, polyethylene glycol and benzyl alcohol (for injection or liquid preparations); and petrolatum (e.g., VASELINE), vegetable oil, animal fat and polyethylene glycol (for

externally applicable preparations). In addition, if desired, the composition can contain wetting or emulsifying agents, isotonic agents, dissolution promoting agents, stabilizers, colorants, antiseptic agents, soothing agents and the like additives (as usual auxiliary additives to pharmaceutical preparations), pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The therapeutic compositions of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions.

A therapeutic composition typically contains an amount of a therapeutic nucleotide sequence of the present invention sufficient to deliver a therapeutically effective amount to the target tissue, typically an amount of at least 0.1 weight percent to about 90 weight percent of therapeutic nucleotide sequence per weight of total therapeutic composition. A weight percent is a ratio by weight of therapeutic nucleotide sequence to total composition. Thus, for example, 0.1 weight percent is 0.1 grams of DNA segment per 100 grams of total composition.

The therapeutic nucleotide compositions comprising synthetic oligonucleotide sequences of the present invention can be prepared using any suitable method, such as, the phosphotriester or phosphodiester methods. See Narang et al., Meth. Enzymol., 68:90,

(1979); U.S. Patent No. 4,356,270; and Brown et al., Meth. Enzymol., 68:109, (1979), the disclosures of which are incorporated by reference herein.

For therapeutic oligonucleotides sequence compositions in which a family of variants is preferred, the synthesis of the family members can be conducted simultaneously in a single reaction vessel, or can be synthesized independently and later admixed in preselected molar ratios. For simultaneous synthesis, the nucleotide residues that are conserved at preselected positions of the sequence of the family member can be introduced in a chemical synthesis protocol simultaneously to the variants by the addition of a single preselected nucleotide precursor to the solid phase oligonucleotide reaction admixture when that position number of the oligonucleotide is being chemically added to the growing oligonucleotide polymer. The addition of nucleotide residues to those positions in the sequence that vary can be introduced simultaneously by the addition of amounts, preferably equimolar amounts, of multiple preselected nucleotide precursors to the solid phase oligonucleotide reaction admixture during chemical synthesis. For example, where all four possible natural nucleotides (A,T,G and C) are to be added at a preselected position, their precursors are added to the oligonucleotide synthesis reaction at that step to simultaneously form four variants.

This manner of simultaneous synthesis of a family of related oligonucleotides has been previously described for the preparation of "Degenerate Oligonucleotides" by Ausubel et al. (Current Protocols in Molecular Biology, Suppl. 8. p.2.11.7, John Wiley & Sons, Inc., New York (1991)), and can readily be applied to the preparation of the therapeutic oligonucleotide compositions described herein.

Nucleotide bases other than the common four nucleotides (A,T,G or C), or the RNA equivalent nucleotide uracil (U), can also be used in the present invention. For example, it is well known that inosine (I) is capable of hybridizing with A, T and G, but not C. Examples of other useful nucleotide analogs are known in the art; many may be found listed in 37 C.F.R. §1.822.

Thus, where all four common nucleotides are to occupy a single position of a family of oligonucleotides, that is, where the preselected therapeutic nucleotide composition is designed to contain oligonucleotides that can hybridize to four sequences that vary at one position, several different oligonucleotide structures are contemplated. The composition can contain four members, where a preselected position contains A,T,G or C. Alternatively, the

composition can contain two members, where a preselected position contains I or C, and has the capacity to hybridize at that position to all four possible common nucleotides.' Finally, other nucleotides may be included at the preselected position that have the capacity to hybridize in a non-destabilizing manner with more than one of the common nucleotides in a manner similar to inosine.

3. Expression Vector Systems

The introduction of exogenous DNA into eucaryotic cells has become one of the most powerful tools of the molecular biologist. The term "exogenous" encompasses any therapeutic composition of this invention which is administered by the therapeutic methods of this invention. Thus, "exogenous" may also be referred to herein as "foreign," "non-native," and the like. The methods of this invention preferably require efficient delivery of the DNA into the nucleus of the recipient cell and subsequent identification of cells that are expressing the foreign DNA.

A widely-used plasmid is pBR322, a vector whose nucleotide sequence and endonuclease cleavage sites are well known. Various other useful plasmid vectors are described in the Examples that follow.

A vector of the present invention comprises a nucleic acid (preferably DNA) molecule capable of autonomous replication in a cell and to which a DNA segment, e.g., a gene or polynucleotide, can be operatively linked so as to bring about replication of the attached segment. In the present invention, one of the nucleotide segments to be operatively linked to vector sequences encodes at least a portion of a therapeutic nucleic acid molecule -- in effect, a nucleic acid sequence that encodes one or more therapeutic proteins or polypeptides, or fragments thereof.

In various embodiments, the entire peptide-coding sequence of the therapeutic gene is inserted into the vector and expressed; however, it is also feasible to construct a vector which also includes some non-coding sequences as well. Preferably, however, non-coding sequences are excluded. Alternatively, a nucleotide sequence for a soluble form of a polypeptide may be utilized. Another preferred therapeutic viral vector includes a nucleotide sequence encoding at least a portion of a therapeutic nucleotide sequence operatively linked to the vector for expression. As used herein with regard to DNA sequences or segments, the phrase

"operatively linked" generally means the sequences or segments have been covalently joined into one piece of DNA, whether in single or double stranded form.

The choice of viral vector into which a therapeutic nucleotide sequence of this invention is operatively linked depends directly, as is well known in the art, on the functional properties desired, e.g., vector replication and protein expression, and the host cell to be transformed -- these being limitations inherent in the art of constructing recombinant DNA molecules. Although certain adenovirus serotypes are recited herein in the form of specific examples, it should be understood that the present invention contemplates the use of *any* adenovirus serotype, including hybrids and derivatives thereof. As one will observe, it is not unusual or outside the scope of the present invention to utilize nucleotide and/or amino acid residue sequences of two or more serotypes in constructs, compositions and methods of the invention.

As one of skill in the art will note, in various embodiments of the present invention, different "types" of vectors are disclosed. For example, one "type" of vector is used to deliver particular nucleotide sequences into a packaging cell line, with the intent of having said sequences stably integrate into the cellular genome; these "types" of vectors are generally identified herein as complementing plasmids. A further "type" of vector described herein carries or delivers nucleotide sequences in or into a cell line (e.g., a packaging cell line) for the purpose of propagating therapeutic viral vectors of the present invention; hence, these vectors are generally referred to herein as delivery plasmids. A third "type" of vector described herein is utilized to carry nucleotide sequences encoding therapeutic proteins or polypeptides to specific cells or cell types in a subject in need of treatment; these vectors are generally identified herein as therapeutic viral vectors or Ad-derived vectors.

In one embodiment, the directional ligation means is provided by nucleotides present in the upstream nucleotide sequence, downstream nucleotide sequence, or both. In another embodiment, the sequence of nucleotides adapted for directional ligation comprises a sequence of nucleotides that defines multiple directional cloning means. Where the sequence of nucleotides adapted for directional ligation defines numerous restriction sites, it is referred to as a multiple cloning site.

A translatable nucleotide sequence is a linear series of nucleotides that provide an uninterrupted series of at least 8 codons that encode a polypeptide in one reading frame.

Preferably, the nucleotide sequence is a DNA sequence. The vector itself may be of any suitable type, such as a viral vector (RNA or DNA), naked straight-chain or circular DNA, or a vesicle or envelope containing the nucleic acid material and any polypeptides that are to be inserted into the cell.

A preferred viral vector in which therapeutic nucleotide compositions of this invention are present is derived from adenovirus (Ad). It is also desirable that the vector contain a promoter sequence. As taught herein, viral vectors of this invention may be designed and constructed in such a way that they specifically target a preselected recipient cell type, depending on the nature of therapy one seeks to administer. Methods of making and using therapeutic viral vectors that target specific cells are further described in the Examples that follow.

Novel vectors and compositions may also be designed and prepared to preferentially target cells that might not otherwise be targeted by wild-type adenovirus virions. For example, in order to target non-epithelial cells, one following the teachings of the present specification may be able to prepare a therapeutic vector including a nucleotide sequence encoding a foreign protein, polypeptide or other ligand directed to a non-epithelial cell or to a different receptor than that generally targeted by a particular adenovirus. Examples of useful ligands directed to specific receptors (identified in parentheses) include the V3 loop of HIV gp120 (CD4); transferrin (transferrin receptor); LDL (LDL receptors); and deglycosylated proteins (asialoglycoprotein receptor). Various useful ligands which may be added to adenovirus fiber -- and methods for preparing and attaching same -- are set forth in published International App. No. WO95/26412, the disclosures of which are incorporated by reference herein.

Useful ligands which may be encoded by a foreign nucleotide sequence contained within a viral vector of the present invention, or which may be linked to proteins or polypeptides expressed thereby after said vectors are administered to a subject, also include antibodies and attachment sequences, as well as receptors themselves. For example, antibodies to cell receptor molecules such as integrins and the like, MHC Class I and Class II, asialoglycoprotein receptor, transferrin receptors, LDL receptors, CD4, and CR2 are but a few which are useful according to the present invention. It is also understood that the ligands typically bound by receptors, as well as analogs to those ligands, may be used as cellular targeting agents, as disclosed herein.

E. Therapeutic Methods

The vectors of the present invention are particularly suited for gene therapy. Thus, various therapeutic methods are contemplated by the present invention.

For example, it has now been discovered that Ad-derived viral vectors are capable of delivering a therapeutic nucleotide sequence to a specific cell or tissue, thereby expanding and enhancing treatment options available in numerous conditions in which more conventional therapies are of limited efficacy. Accordingly, methods of gene therapy utilizing these vectors are within the scope of the invention. Vectors are typically purified and then an effective amount is administered *in vivo* or *ex vivo* into the subject.

For example, the compositions may be used prophylactically or therapeutically *in vivo* to disrupt HIV infection and mechanisms of action by inhibiting gene expression or activation, via delivery of antisense HIV sequences or ribozymes to T cells or monocytes. Using methods of the present invention, one may target therapeutic viral vectors as disclosed herein to specific cells and tissues, including hematopoietic cells, as infection of such cells appears to be mediated by distinct integrins to which viral vectors of the present invention may readily be targeted. (See, e.g., Huang, et al., *J. Virol.* 70: 4502-8 (1996).)

Other useful therapeutic nucleotide sequences include antisense nucleotide sequences complementary to EBV EBNA-1 gene. Use of such therapeutic sequences may remediate or prevent latent infection of B cells with EBV. As discussed herein and in the Examples below, targeting and delivery may be accomplished via the use of various ligands, receptors, and other appropriate targeting agents.

Thus, in one embodiment, a therapeutic method of the present invention comprises contacting the cells of a subject infected with EBV or HIV with a therapeutically effective amount of a pharmaceutically acceptable composition comprising a therapeutic nucleotide sequence of this invention. In a related embodiment, the contacting involves introducing the therapeutic nucleotide sequence composition into cells having an EBV or HIV-mediated infection.

Methods of gene therapy are well known in the art (see, e.g., Larrick and Burck, Gene Therapy: Application of Molecular Biology, Elsevier Science Publ. Co., Inc., New York, NY (1991); Kriegler, Gene Transfer and Expression: A Laboratory Manual, W. H. Freeman and Company, New York (1990)). The term "subject" should be understood to include any animal

-- particularly mammalian -- patient, such as any murine, rat, bovine, porcine, canine, feline, equine, ursine, or human patient.

When the foreign gene carried in the vector encodes a tumor suppressor gene or another anti-tumor protein, the vector is useful to treat or reduce hyperproliferative cells in a subject, to inhibit tumor proliferation in a subject or to ameliorate a particular, related pathology. Pathologic hyperproliferative cells are characteristic of various disease states, such as thyroid hyperplasia, psoriasis, eczema, benign prostatic hypertrophy, Li-Fraumeni syndrome including breast cancer, sarcomas and other neoplasms, bladder cancer, colon cancer, lung cancer, various leukemias, and lymphomas.

Non-pathologic hyperproliferative cells are found, for example, in cells associated with wound repair. Pathologic hyperproliferative cells, however, characteristically exhibit loss of contact inhibition and a decline in their ability to selectively adhere which implies a change in the surface properties of the cell and a further breakdown in intercellular communication. These changes include stimulation to divide and the ability to secrete proteolytic enzymes.

The present invention also contemplates methods of depleting suitable samples of pathologic mammalian hyperproliferative cells contaminating hematopoietic precursors during bone marrow reconstitution via the introduction of a wild-type tumor suppressor gene into the cell preparation using a vector of this invention. As used herein, a suitable sample is defined as a heterogeneous cell preparation obtained from a patient, e.g., a mixed population of cells containing both phenotypically normal and pathogenic cells.

Administration includes -- but is not limited to -- the introduction of therapeutic agents of the present invention into a cell or subject via various means, including direct injection, intravenously, intraperitoneally, via intra-tumor injection, via aerosols, or topically. Therapeutic agents as disclosed herein may also be combined for administration of an effective amount of the agents with a pharmaceutically-acceptable carrier, as described herein.

As used herein, "effective amount" generally means the amount of vector (or proteins produced/released thereby) which achieves a positive outcome in the subject to whom the vector is administered. The total volume administered will necessarily vary depending on the mode of administration, as those of skill in the relevant art will appreciate, and dosages may vary as well.

The dose of a biologic vector is somewhat complex and may be described in terms of the concentration (in plaque-forming units per milliliter (pfu/ml)), the total dose (in pfus), and the estimated number of vectors administered per cell (the estimated multiplicity of infection or MOI). Thus, if a vector is administered via infusion -- say, across nasal epithelium -- at a constant total volume, the respective concentration, etc. may be described as follows:

Concentration (pfu/ml)	Volume (ml)	Dose (pfu)	Estimated MOI
10^7	2	2×10^7	1
10^8	2	2×10^8	10
10^9	2	2×10^9	100
10^{10}	2	2×10^{10}	1000

In general, when adenoviral vectors are administered via infusion across the nasal epithelium, administered amounts producing an estimated MOI of about 10 or greater are much more effective than lower dosages. (See, e.g., Knowles, et al., New Eng. J. Med. 333: 823-831 (1995).) Similarly, when direct injection is the preferred treatment modality -- e.g., direct injection of a viral vector into a tumor -- doses of 1×10^9 pfu or greater are generally preferred. (See, e.g., published International App. No. WO95/11984.)

Thus, depending on the mode of administration, an effective amount administered in a single dose preferably contains from about 10^6 to about 10^{15} infectious units. A typical course of treatment would be one such dose per day over a period of five days. As those of skill in the art will appreciate, an effective amount may vary depending on (1) the pathology or other condition to be treated, (2) the status and sensitivity of the patient, and (3) various other factors well known to those of skill in the art, such as the patient's tolerance to other courses of treatment that may have been applied previously. Thus, those of skill in the art may easily and precisely determine effective amounts of the agents/vectors of the present invention which may be administered to a particular patient, based on their understanding of and evaluation of such factors.

The present invention also contemplates methods of ameliorating pathologies characterized by hyperproliferative cells or genetic defects in a subject, by administering to the subject an effective amount of a vector as described herein. Such vectors preferably contain a foreign gene encoding a gene product (e.g. polypeptide or protein) having the ability to ameliorate the pathology, under suitable conditions. As used herein, the term "genetic defect" means any disease, condition or abnormality which results from inherited factors, e.g. Huntington's Disease, Tay-Sachs Disease, or Sickle Cell Disease.

The present invention further provides methods for reducing the proliferation of tumor cells in a subject by introducing into the tumor mass an effective amount of an adenoviral expression vector containing an anti-tumor gene other than a tumor suppressor gene. The anti-tumor gene can encode, for example, thymidine kinase (TK). An effective amount of a therapeutic agent is then administered to the subject; the therapeutic agent, in the presence of the anti-tumor gene, is toxic to the cell.

Using thymidine kinase as exemplary, the therapeutic agent is a thymidine kinase metabolite such as ganciclovir (GCV), 6-methoxypurine arabinonucleoside (araM), or a functional equivalent thereof. Both the thymidine kinase gene and the thymidine kinase metabolite must be used concurrently in order to exert a toxic effect on the host cell. In the presence of the TK gene, GCV is phosphorylated and becomes a potent inhibitor of DNA synthesis, whereas araM is converted to the cytotoxic anabolite araATP. Thus, the precise method of action or synergism is not relevant to therapeutic efficacy; what is relevant is the fact that the concurrent use of appropriate genes and therapeutic agents may effectively ameliorate a specific disease condition.

Another useful example contemplates use of a vector of the present invention which expresses the enzyme cytosine deaminase. Such a vector could be used in conjunction with administration of the drug 5-fluorouracil (Austin and Huber, *Mol. Pharm.* 43: 380-387 (1993)) or the recently-described *E. coli* Deo⁻ gene in combination with 6-methyl-purine-2'-deoxyribonucleoside (Sorscher et al., *Gene Therapy* 1: 233-238 (1994)).

As with the use of the tumor suppressor genes described previously, the use of other anti-tumor genes, either alone or in combination with the appropriate therapeutic agent, provides a treatment for the uncontrolled cell growth or proliferation characteristic of tumors and malignancies. Thus, the present invention provides therapies to halt the uncontrolled

cellular growth in a patient, thereby alleviating the symptoms or the disease or cachexia present in the patient. The effect of this treatment includes, but is not limited to, prolonged survival time of the patient, reduction in tumor mass or burden, apoptosis of tumor cells, or the reduction in the number of circulating tumor cells. Means of quantifying the beneficial effects of this therapy are well known to those of skill in the art.

The present invention provides a recombinant adenovirus expression vector characterized by the partial or total deletion of one or more adenoviral structural protein genes, such as the gene encoding fiber, which allows the vector to accommodate a therapeutic, foreign nucleic acid sequence encoding a functional foreign polypeptide, protein, or biologically active fragment thereof. For example, such a functional polypeptide moiety may be a suicide gene or a functional equivalent thereof, of which the anti-cancer gene TK is but one example. TK genes, when expressed, produce a gene product which is lethal to the cell, particularly in the presence of GCV. One source of the TK gene is the herpes simplex virus (HSV), albeit other sources are known as well and may be used as taught herein. The TK gene may readily be obtained from HSV by methods well known to those of skill in the art. For example, the plasmid pMLBTK in *E. coli* HB101 (from ATCC #39369) is a source of the HSV-1 TK gene, which may be used as disclosed herein. (See, e.g., published International application No. WO 95/11984, the disclosures of which are incorporated by reference herein.)

A therapeutic gene sequence may be introduced into a tumor mass by combining the adenoviral expression vector with a suitable pharmaceutically acceptable carrier. Introduction can be accomplished, for example, via direct injection of the recombinant Ad vector into the tumor mass. In the specific case of a cancer such as hepatocellular carcinoma (HCC), direct injection into the hepatic artery can be used for delivery, because most HCCs derive their circulation from this artery. Similar techniques of administration may be applied to other specific types of tumors and malignancies, as is known to those of skill in the art.

A method of tumor-specific delivery of a tumor-suppressor gene is accomplished by contacting target tissue in a subject with an effective amount of a recombinant Ad-derived vector of this invention. In the case of anti-tumor therapy, the gene is intended to encode an anti-tumor agent, such as a functional tumor suppressor gene product or suicide gene product. The term "contacting" is intended to encompass any delivery method for the efficient transfer of the vector, such as via intra-tumoral injection.

In another example, adenovirus vectors of the present invention can be used to transfer genes to central nervous system (CNS) tumors *in vivo*. Using stereotactic delivery, Ad-derived vectors can transfer genes into the CNS intended for tumor therapy. For example, Badie, et al. (*Neurosurgery* 35(5): 910-916 (1994), incorporated by reference herein) reported that 50% and 90% transduction at vector titers of approximately 10^7 and 10^8 plaque-forming units/ml (pfu/ml) were observed in *in vitro* experiments. In their *in vivo* studies using appropriate animal brain tumor models, titers above 10^7 were observed to have a cytopathic effect; more than 50% reduction in tumor cell growth was noted at 10^8 pfu/ml; no toxic effects were noted when titers as high as 10^{10} pfu/ml were injected into the brain tissue of subject animals (*Id.*). Thus, the use of titers greater than 10^7 pfu/ml appear appropriate when challenging CNS tumors.

The present invention also contemplates methods for determining the efficacy of the within-disclosed therapeutic compositions and methods. One such method for confirming efficacy utilizes the human/SCID (severe combined immunodeficient) mouse model of EBV-induced LPD (lymphoproliferative disease) to ascertain whether EBV-antisense therapeutic nucleotide sequences block tumor formation. (See, e.g., Pisa, et al., *Blood* 79: 173-179 (1992); Rowe, et al., *Curr. Top. Microbiol. Immunol.* 166: 325 (1990); and Cannon, et al., *J. Clin. Invest.* 85: 1333-1337 (1990), the disclosures of which are incorporated by reference herein.)

Finally, the use of Ad vectors of the present invention to prepare medicaments for the treatment, therapy and/or diagnosis of various diseases is also contemplated by this invention. Moreover, other anti-tumor genes may be used in combination with the corresponding therapeutic agent to reduce the proliferation of tumor cells. Such other gene-and-therapeutic-agent combinations are known to those of skill in the art and may be applied as taught herein.

F. Construction of Therapeutic Viral Vectors for Gene Delivery

For *in vitro* gene transfer, administration is often accomplished by first isolating a selected cell population from a patient such as lung epithelial cells, lymphocytes and the like followed by *in vitro* gene transfer of the therapeutic compositions of this invention and the replacement of the cells into the patient. *In vivo* therapy is also contemplated, e.g., via the administration of therapeutic compositions of this invention by various delivery means. For

example, aerosol administration and administration via subcutaneous, intravenous, intraperitoneal, intramuscular, ocular means and the like are also within the scope of the present invention.

Other gene-delivery methods are also useful in conjunction with the methods, compositions and constructs of the present invention; see, e.g., published International Application No. WO 95/11984, the disclosures of which are incorporated by reference herein.

Similarly, various non-human animals having inserted therein the vectors or transformed cells of this invention. These "transgenic" animals are made using methods well known to those of skill in the art. For example, see U.S. Patent No. 5,175,384 (the disclosures of which are incorporated by reference herein).

The present invention also contemplates various methods of targeting specific cells -- e.g., cells in a subject in need of diagnosis and/or treatment. As discussed herein, the present invention contemplates that the viral vectors and compositions of the present invention may be directed to specific receptors or cells, for the ultimate purpose of delivering those vectors and compositions to specific cells or cell types. The viral vectors and constructs of the present invention are particularly useful in this regard.

In general, adenovirus attachment and uptake into cells are separate but cooperative events that result from the interaction of distinct viral coat proteins with a receptor for attachment and γ integrin receptors for internalization. Adenovirus attachment to the cell surface via the fiber coat proteins has been discovered to be dissociable and distinct from the subsequent step of internalization, and the present invention is able to take advantage of and function in conjunction with these differing receptors.

G. Other Applications

The cell lines, viral vectors and methods of the present invention may also be used for purposes other than the direct administration of therapeutic nucleotide sequences. In one such application, the production of large quantities of biologically active proteins or polypeptides in cells transfected with the within-disclosed viral vectors is contemplated herein. For example, human lymphoblastoid cells may be transfected with an integrative viral vector of the present invention carrying a human hematopoietic growth factor such as the gene for erythropoietin (EPO); cells so transfected are thus able to produce biologically active EPO. (See, e.g., Lopez et al., Gene 148: 285-91 (1994).)

Various other applications and uses of the within-described methods, cell lines, plasmids, vectors, and compositions of the present invention shall become apparent upon closer examination of the Examples that follow.

EXAMPLES

The following examples are intended to illustrate, but not limit, the present invention. As such, the following description provides details of the manner in which particular embodiments of the present invention may be made and used. This description, while exemplary of the present invention, is not to be construed as specifically limiting the invention. Variations and equivalents, now known or later developed, which would be within the understanding and technical competence of one skilled in this art are to be considered as falling within the scope of this invention.

Example 1

Preparation of Adenovirus Packaging Cell Lines

Cell lines that are commonly used for growing adenovirus are useful as host cells for the preparation of adenovirus packaging cell lines. Preferred cells include 293 cells, an adenovirus-transformed human embryonic kidney cell line obtained from the ATCC, having Accession Number CRL 1573; HeLa, a human epithelial carcinoma cell line (ATCC Accession Number CCL 2); A549, a human lung carcinoma cell line (ATCC Accession Number CCL 1889); and the like epithelial-derived cell lines. As a result of the adenovirus transformation, the 293 cells contain the E1 early region regulatory gene. All cells were maintained in complete DMEM + 10% fetal calf serum unless otherwise noted.

The cell lines of this invention allow for the production and propagation of novel adenovirus-based gene delivery vectors having deletions in preselected gene regions by cellular complementation of adenoviral genes. To provide the desired complementation of such deleted adenoviral genomes in order to generate a novel viral vector of the present invention, plasmid vectors that contain preselected functional units were designed as described herein. Such units include but are not limited to E1 early region, ????? and the viral fiber gene. The preparation of plasmids providing such complementation, thereby being "complementary plasmids or constructs", that are stably inserted into host cell chromosomes are described below.

A. Preparation of an E4-Expressing Plasmid for Complementation of E4-Gene-Deleted Adenoviruses

The viral E4 regulatory region contains a single transcription unit which is alternately spliced to produce several different mRNAs. The E4-expressing plasmid prepared as described herein and used to transfect the 293 cell line contains the entire E4 transcriptional unit as shown in Figure 1. A DNA fragment extending from 175 nucleotides upstream of the E4 transcriptional start site including the natural E4 promoter to 153 nucleotides downstream of the E4 polyadenylation signal including the natural E4 terminator signal, corresponding to nucleotides 32667-35780 of the adenovirus type 5 (hereinafter referred to as Ad5) genome as described in Chroboczek et al. (*Virol.*, 186:280-285 (1992), GenBank Accession Number M73260), was amplified from Ad5 genomic DNA, obtained from the ATCC, via the polymerase chain reaction (PCR). Sequences of the primers used were 5'CGGTACACAGAATTCAGGAGACACAACTCC3' (forward or 5' primer referred to as E4L) (SEQ ID NO 1) and 5'GCCTGGATCCGGAAAGTTACGTAACGTGGAAAAC3' (SEQ ID NO 2) (backward or 3' primer referred to as E4R). To facilitate cloning of the PCR fragment, these oligonucleotides were designed to create novel sites for the restriction enzymes EcoRI and BamHI, respectively, as indicated with underlined nucleotides. DNA was amplified via PCR using 30 cycles of 92 C for 1 minute, 50 C for 1 minute, and 72 C for 3 minutes resulting in amplified full-length E4 gene products.

The amplified DNA E4 products were then digested with EcoRI and BamHI for cloning into the compatible sites of pBluescript/SK+ by standard techniques to create the plasmid pBS/E4. A 2603 base pair (bp) cassette including the herpes simplex virus thymidine kinase promoter, the hygromycin resistance gene, and the thymidine kinase polyadenylation signal was excised from the plasmid pMEP4 (Invitrogen, San Diego, CA) by digestion with FspI followed by addition of BamHI linkers (5'CGCGGATCCCGCG3') (SEQ ID NO 3) for subsequent digestion with BamHI to isolate the hygromycin-containing fragment.

The isolated BamHI-modified fragment was then cloned into the BamHI site of pBS/E4 containing the E4 region to create the plasmid pE4/Hygro containing 8710 bp (Figure 2). The pE4/Hygro plasmid has been deposited with the ATCC as described in Example 3. The complete nucleotide sequence of pE4/Hygro is listed in SEQ ID NO 4. Position number 1 of the linearized vector corresponds to approximately the middle portion of the pBS/SK+

backbone as shown in Figure 2 as a thin line between the 3' BamHI site in the hygromycin insert and the 3' EcoRI site in the E4 insert. The 5' and 3' ends of the E4 gene are located at respective nucleotide positions 3820 and 707 of SEQ ID NO 4 while the 5' and 3' ends of the hygromycin insert are located at respective nucleotide positions 3830 and 6470. In the clone that was selected for use, the E4 and hygromycin resistance genes were divergently transcribed.

B. Preparation of a Fiber-Expressing Plasmid for Complementation of Fiber-Gene-Deleted Adenoviruses

To prepare a fiber-encoding construct, primers were designed to amplify the fiber coding region from Ad5 genomic DNA with the addition of unique BamHI and NotI sites at the 5' and 3' ends of the fragment, respectively. The Ad5 nucleotide sequence is available with the GenBank Accession Number M18369. The 5' and 3' primers had the respective nucleotide sequences of 5' ATGGGATCCAAGATGAAGCGCGCAAGACCG3' (SEQ ID NO 5) and 5' CATAACCGCGCCGCTTCTTATTCTTGGGC3' (SEQ ID NO 6), where the inserted BamHI and NotI sites are indicated by underlining. The 5' primer also contained a nucleotide substitution 3 nucleotides 5' of the second ATG codon (C to A) that is the initiation site. The nucleotide substitution was included so as to improve the consensus for initiation of fiber protein translation.

The amplified DNA fragment was inserted into the BamHI and NotI sites of pcDNA 3 (Invitrogen) to create the plasmid designated pCDNA3/Fiber having 7148 bp, the plasmid map of which is shown in Figure 3. The parent plasmid contained the CMV promoter, the bovine growth hormone (BHG) terminator and the gene for conferring neomycin resistance. The viral sequence included in this construct corresponds to nucleotides 31040-32791 of the Ad5 genome.

The complete nucleotide sequence of pCDNA3/Fiber is listed in SEQ ID NO 7 where the nucleotide position 1 corresponds to approximately the middle of the pcDNA 3 vector sequence. The 5' and 3' ends of the fiber gene are located at respective nucleotide positions 916 with ATG and 2661 with TAA.

To enhance expression of fiber protein by the constitutive CMV promoter provided by the pcDNA vector, a BglII fragment containing the tripartite leader (TPL) of adenovirus type 2 was excised from pRD112a (Sheay et al., BioTechniques, 15:856-862 (1993) and inserted into

the BamHI site of pCDNA3/Fiber to create the plasmid pCLF having 7469 bp, the plasmid map of which is shown in Figure 4. The adenovirus tripartite leader sequence, present at the 5' end of all major late adenoviral mRNAs as described by Logan et al., Proc. Natl. Acad. Sci. USA, 81:3655-3659 (1984) and Berkner, BioTechniques, 6:616-629 (1988), is encoded by three spatially separated exons corresponding to nucleotide positions 6071-6079 (the 3' end of the first leader segment), 7101-7172 (the entire second leader segment), and 9634-9721 (the third leader segment) in the adenovirus type 2 genome. The tripartite sequence, however, also shows correspondence with the Ad5 leader sequence having three spatially separated exons corresponding to nucleotide positions 6081-6089 (the 3' end of the first leader segment), 7111-7182 (the entire second leader segment), and 9644-9845 (the third leader segment and sequence downstream of that segment). The corresponding cDNA sequence of the tripartite leader sequence present in pCLF is listed in SEQ ID NO 8 bordered by BamHI/BglII 5' and 3' sites at respective nucleotide positions 907-912 to 1228-1233.

The pCLF plasmid has been deposited with the ATCC as described in Example 3. The complete nucleotide sequence of pCLF is listed in SEQ ID NO 8 where the nucleotide position 1 corresponds to approximately the middle of the pcDNA 3 parent vector sequence. The 5' and 3' ends of the Ad5 fiber gene are located at respective nucleotide positions 1237-1239 with ATG and 2980-2982 with TAA. The rest of the vector construct has been previously described above.

C. Generation of an Adenovirus Packaging Cell Line Carrying Plasmids Encoding Functional E4 and Fiber Proteins

The 293 cell line was selected for preparing the first adenovirus packaging line as it already contains the E1 gene as prepared by Graham et al., J. Gen. Virol., 36:59-74 (1977) and as further characterized by Spector, Virol., 130:533-538 (1983). Before electroporation, 293 cells were grown in RPMI medium + 10% fetal calf serum. Four x 10⁶ cells were electroporated with 20 µg each of pE4/Hygro DNA and pCLF DNA using a BioRad GenePulser and settings of 300 V, 25 µF. DNA for electroporation was prepared using the Qiagen system according to the manufacturer's instructions (Bio-Rad, Richmond, CA).

Following electroporation, cells were split into fresh complete DMEM + 10% fetal calf serum containing 200 µg/ml Hygromycin B (Sigma, St. Louis, MO).

From expanded colonies, genomic DNA was isolated using the "MICROTURBOGEN" system (Invitrogen) according to manufacturer's instructions. The presence of integrated E4 DNA was assessed by PCR using the primer pair E4R and ORF6L (5'TGCTTAAGCGGCCGCGAAGGAGAAGTCC3') (SEQ ID NO 9), the latter of which is a 5' forward primer near adenovirus 5 open reading frame 6. Refer to Figure 1 for position of the primers relative to the E4 genes.

One clone, designated 211, was selected exhibiting altered growth properties relative to that seen in parent cell line 293. The 211 clone contained the expected product, indicating the presence of inserted DNA corresponding to most, if not all, of the E4 fragment contained in the pE4/Hygro plasmid. The 211 cell line has been deposited with the ATCC as described in Example 3. This line was further evaluated by amplification using the primer pair E4L/E4R described above, and a product corresponding to the full-length E4 insert was detected. Genomic Southern blotting was performed on DNA restricted with EcoRI and BamHI. The E4 fragment was then detected at approximately one copy/genome compared to standards with the EcoRI/BamHI E4 fragment as cloned into pBS/E4 for use as a labeled probe with the Genius system according to manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). In DNA from the 211 cell line, the expected labeled internal fragment pE4/Hygro hybridized with the isolated E4 sequences. In addition, the probe hybridized to a larger fragment which may be the result of a second insertion event (Figure 5).

Although the 211 cell line was not selected by neomycin resistance, thus indicating the absence of fiber gene, to confirm the lack of fiber gene, the 211 cell line was analyzed for expression of fiber protein by indirect immunofluorescence with an anti-fiber polyclonal antibody and a FITC-labeled anti-rabbit IgG (KPL) as secondary. No immunoreactivity was detected. Therefore, to generate 211 clones containing recombinant fiber genes, the 211 clone was expanded by growing in RPMI medium and subjected to additional electroporation with the fiber-encoding pCLF plasmid as described above.

Following electroporation, cells were plated in DMEM + 10% fetal calf serum and colonies were selected with 200 µg/ml G418 (Gibco, Gaithersburg, MD). Positive cell lines remained hygromycin resistant. These candidate sublines of 211 were then screened for fiber protein expression by indirect immunofluorescence as described above. The three sublines screened, 211A, 211B and 211R, along with a number of other sublines, all exhibited nuclear

staining qualitatively comparable to the positive control of 293 cells infected with AdRSV gal (1 pfu/cell) and stained 24 hours post-infection.

Lines positive for nuclear staining in this assay were then subjected to Western blot analysis under denaturing conditions using the same antibody. Several lines in which the antibody detected a protein of the expected molecular weight (62 kd for the Ad5 fiber protein) were selected for further study including 211A, 211B and 211R. The 211A cell line has been deposited with ATCC as described in Example 3.

Western blot analysis using soluble nuclear extracts from these three cell lines and a seminative electrophoresis system demonstrated that the fiber protein expressed is in the functional trimeric form characteristic of the native fiber protein as shown in Figure 6. The predicted molecular weight of a trimerized fiber is 186 kd. The lane marked 293 lacks fiber while the sublines contain detectable fiber. Under denaturing conditions, the trimeric form was destroyed resulting in detectable fiber monomers as shown in Figure 6. Those clones containing endogenous E1, newly expressed recombinant E4 and fiber proteins were selected for use in complementing adenovirus gene delivery vectors having the corresponding adenoviral genes deleted as described in Example 2.

D. Preparation of an E1-Expressing Plasmid for Complementation of E1-Gene-Deleted Adenoviruses

In order to prepare adenoviral packaging cell lines other than those based on the E1-gene containing 293 cell line as described in Example 1C above, plasmid vectors containing E1 alone or in various combinations with E4 and fiber genes are constructed as described below.

The region of the adenovirus genome containing the E1a and E1b gene is amplified from viral genomic DNA by PCR as previously described. The primers used are E1L, the 5' or forward primer, and E1R, the 3' or backward primer, having the respective nucleotide sequences 5'CCGAGCTAGCGACTGAAAATGAG3' (SEQ ID NO 10) and 5'CCTCTCGAGGAGACAGCAAGACAC3' (SEQ ID NO 11). The E1L and E1R primers include the respective restriction sites NheI and XhoI as indicated by the underlines. The sites are used to clone the amplified E1 gene fragment into the NheI/XhoI sites in pMAM commercially available from Clontech (Palo Alto, CA) to form the plasmid pDEX/E1 having 11152 bp, the plasmid map of which is shown in Figure 7.

The complete nucleotide sequence of pDEX/E1 is listed in SEQ ID NO 12 where the nucleotide position 1 corresponds to approximately 1454 nucleotides from the 3' end of the pMAM backbone vector sequence. The pDEX/E1 plasmid includes nucleotides 552 to 4090 of the adenovirus genome positioned downstream (beginning at nucleotide position 1460 and ending at 4998 in the pDEX/E1 plasmid) of the glucocorticoid-inducible mouse mammary tumor virus (MMTV) promoter of pMAM. The pMAM vector contains the *E. coli* *gpt* gene that allows stable transfecants to be isolated using hypoxanthine/aminopterin/thymidine (HAT) selection. The pMAM backbone occupies nucleotide positions 1-1454 and 5005-11152 of SEQ ID NO 12.

E. Generation of an Adenovirus Packaging Cell Line Carrying Plasmids Encoding Functional E1, and Fiber Proteins

To create separate adenovirus packaging cell lines equivalent to that of the 211 sublines, 211A, 211B and 211R, as described in Example 1C, alternative cell lines lacking adenoviral genomes are selected for transfection with the plasmid constructs as described below. Acceptable host cells include A549, HeLa, Vero and the like cell lines as described in Example 1. The selected cell line is transfected with the separate plasmids, pDEX/E1 and pCLF, respectively for expressing E1, and fiber complementary proteins. Following transfection procedures as previously described, clones containing stable insertions of the two plasmids are isolated by selection with neomycin and HAT. Integration of full-length copy of the E1 gene is assessed by PCR amplification from genomic DNA using the primer set E1L/E1R, as described above. Functional insertion of the fiber gene is assayed by staining with the anti-fiber antibody as previously described.

The resultant stably integrated cell line is then used as a packaging cell system to complement adenoviral gene delivery vectors having the corresponding adenoviral gene deletions as described in Example 2.

F. Preparation of a Plasmid Containing Two or More Adenoviral Genes for Complementing Gene-Deleted Adenoviruses

The methods described in the preceding Examples rely on the use of two plasmids, pE4/Hygro and pCLF, or, pCLF and pDEX/E1 for generating adenoviral cell packaging systems. In alternative embodiments contemplated for use with the methods of this invention, complementing plasmids containing two or more adenoviral genes for expressing of encoded

proteins in various combinations are also prepared as described below. The resultant plasmids are then used in various cell systems with delivery plasmids having the corresponding adenoviral gene deletions. The selection of packaging cell, content of the delivery plasmids and content of the complementing plasmids for use in generating recombinant adenovirus viral vectors of this invention thus depends on whether other adenoviral genes are deleted along with the adenoviral fiber gene, and, if so, which ones.

1. Preparation of a Complementing Plasmid Containing Fiber and E1 Adenoviral Genes

A DNA fragment containing sequences for the CMV promoter, adenovirus tripartite leader, fiber gene and bovine growth hormone terminator is amplified from pCLF prepared in Example 1B using the forward primer 5'GACGGATCGGGAGATCTCC3' (SEQ ID NO 13), that anneals to the nucleotides 1-19 of the pCDNA3 vector backbone in pCLF, and the backward primer 5'CCGCCTCAGAAGCCATAGAGCC3' (SEQ ID NO 14) that anneals to nucleotides 1278-1257 of the pCDNA3 vector backbone. The fragment is amplified as previously described and then cloned into the pDEX/E1 plasmid, prepared in Example 1D. For cloning in the DNA fragment, the pDEX/E1 vector is first digested with NdeI, that cuts at a unique site in the pMAM vector backbone in pDEX/E1, then the ends are repaired by treatment with bacteriophage T4 polymerase and dNTPs.

The resulting plasmid containing E1 and fiber genes, designated pE1/Fiber, provides both dexamethasone-inducible E1 function as described for DEX/E1 and expression of Ad5 fiber protein as described above. A schematic plasmid map of pE1/Fiber, having 14455 bp, is shown in Figure 8.

The complete nucleotide sequence of pE1/Fiber is listed in SEQ ID NO 15 where the nucleotide position 1 corresponds to approximately to 1459 nucleotides from the 3' end of the parent vector pMAM sequence. The 5' and 3' ends of the Ad5 E1 gene are located at respective nucleotide positions 1460 and 4998 followed by pMAM backbone and then separated from the Ad5 fiber from pCLF by the filled-in blunt ended NdeI site. The 5' and 3' ends of the pCLF fiber gene fragment are located at respective nucleotide positions 10922-14223 containing elements as previously described for pCLF.

The resultant pE1/Fiber plasmid is then used to complement one or more delivery plasmids expressing E1 and fiber.

The pE1/Fiber construct is then used to transfect a selected host cell as described in Example 1E to generate stable chromosomal insertions preformed as previously described followed by selection on HAT medium. The stable cells are then used as packaging cells as described in Example 2.

2) Preparation of a Complementing Plasmid Containing E4 and Fiber Adenoviral Genes

pCLF prepared as described in Example 1B is partially digested with BglII to cut only at the site in the pCDNA3 backbone. The pE4/Hygro plasmid prepared in Example 1A is digested with BamHI to produce a fragment containing E4. The E4 fragment is then inserted into the BamHI site of pCLF to form plasmid pE4/Fiber. The resultant plasmid provides expression of the fiber gene as described for pCLF and E4 function as described for pE4/Hygro.

A schematic plasmid map of pE4/Fiber, having 10610 bp, is shown in Figure 9. The complete nucleotide sequence of pE4/Fiber is listed in SEQ ID NO 16 where the nucleotide position 1 corresponds to approximately 14 bp from the 3' end of the parent vector pCDNA3 backbone sequence. The 5' and 3' ends of the Ad5 E4 gene are located at respective nucleotide positions 21 and 3149 followed by fused BglII/BamHI sites and pCDNA3 backbone including the CMV promoter again followed by BglII/BamHI sites. The adenovirus leader sequence begins at nucleotide position 4051 and extends to 4366 followed by fused BamHI/BglII sites and the 5' and 3' ends of the fiber gene located at respective nucleotide positions 4372 and 6124.

Stable chromosomal insertions of pE4/Fiber in host cells are obtained as described above.

Example 2

Preparation of Adenoviral Gene Delivery Vectors

Using Adenoviral Packaging Cell Lines

Adenoviral delivery vectors of this invention are prepared to separately lack the combinations of E1/fiber and E4/fiber. Such vectors are more replication-defective than those previously in use due to the absence of multiple viral genes. A preferred adenoviral delivery vector of this invention that is replication competent but only via a non-fiber means is one that only lacks the fiber gene but contains the remaining functional adenoviral regulatory and structural genes. Furthermore, the adenovirus delivery vectors of this invention have a higher capacity for insertion of foreign DNA.

A. Preparation of Adenoviral Gene Delivery Vectors Having Specific Gene Deletions and Methods of Use

To construct the E1/ /fiber deleted viral vector containing the LacZ reporter gene construct, two new plasmids were constructed. The plasmid pΔ E1Bβ gal was constructed as follows. By digestion of pSVβ gal (ProMega Corp., Madison, WI) with VspI, a DNA fragment containing the SV40 regulatory sequences and the *E. coli* -β-galactosidase gene was isolated. The resulting fragment having overhanging ends was then filled in with Klenow fragment of DNA polymerase I in the presence of dNTPs followed by digestion with BamHI. The resulting fragment was cloned into the EcoRV and BamHI sites in the polylinker of pΔ E1sp1B (Microbix Biosystems, Hamilton, Ontario) to form p Δ E1B gal that therefore contained the left end of the adenovirus genome with the Ela region replaced by the LacZ cassette (nucleotides 6690 to 4151) of pSVβ gal. Plasmid DNA was prepared by the alkaline lysis method as described by Birnboim and Doly, Nuc. Acids Res., 7:1513-1523 (1978) from transformed cells used to expand the plasmid. DNA was then purified by CsCl-ethidium bromide density gradient centrifugation.

The second plasmid (pDV44), prepared as described herein, is derived from pBHG10, a vector prepared a described by Bett et al., Proc. Natl. Acad. Sci., USA, 91:8802-8806 (1994) and commercially available from Microbix, which contains an Ad5 genome with the packaging signals at the left end deleted and the E3 region (nucleotides 28133:30818) replaced

by a linker with a unique site for the restriction enzyme *PacI*. An 11.3 kb *BamHI* fragment, which contains the right end of the adenovirus genome, is isolated from pBHG10 and cloned into the *BamHI* site of pBS/SK(+) to create plasmid p11.3 having approximately 14,658 bp. A schematic of the plasmid map is shown in Figure 13. The p11.3 plasmid was then digested with *PacI* and *Sall* to remove the fiber, E4, and inverted terminal repeat (ITR) sequences.

This fragment is replaced with a fragment containing the ITR segments and the E4 gene which is generated by PCR amplification from pBHG10 using the following oligonucleotide sequences (SEQ ID NO 17) (SEQ ID NO 18). These primers incorporate sites for *PacI* and *BamHI*, respectively. Cloning this fragment into the *PacI* and blunt ended *Sall* sites of the p11.3 backbone resulted in a substitution of the fused ITRs, E4 region and fiber gene present in pBHG10, by the ITRs and E4 region alone.

In general, the method for virus production by recombination of plasmids followed by complementation in cell culture involves the isolation of recombinant viruses by cotransfection of any one of the adenovirus packaging cell systems prepared in Example 1, namely 211A, 211B, 211R, A549, Vero cells, and the like, with plasmids carrying sequences corresponding to viral gene delivery vectors.

A selected cell line is plated in dishes and cotransfected with pDV44 and p E1B gal using the calcium phosphate method as described by Bett et al., *Proc. Natl. Acad. Sci., USA*, 91:8802-8806 (1994). Recombination between the overlapping adenovirus sequences in the two plasmids leads to the creation of a full-length viral chromosome where pDV44 and pΔ E1Bβ gal recombine to form a recombinant adenovirus vector having multiple deletions. The deletion of E1 and of the fiber gene from the viral chromosome is compensated for by the sequences integrated into the packaging cell genome, and infectious virus particles are produced. The plaques thus generated are isolated and stocks of the recombinant virus are produced by standard methods.

In a preferred embodiment of this invention, a delivery plasmid is prepared that does not require the above-described recombination events to prepare a therapeutic viral vector having a fiber gene deletion. A single delivery plasmid containing all the adenoviral genome

necessary for packaging but lacking the fiber gene is prepared from plasmid pFG140 containing full-length Ad85 that is commercially available from Microbix. The resultant delivery plasmid referred to as pFG140-f is then used with pCLF stably integrated cells as described above to prepare a therapeutic viral vector lacking fiber. In a preferred aspect of this invention, the fiber gene is replaced with a therapeutic gene of interest for preparing a therapeutic delivery adenoviral vector.

Vectors for the delivery of any desired therapeutic gene are prepared by cloning the gene of interest into the multiple cloning sites in the polylinker of commercially available p E1sp1B (Microbix Biosystems), in an analogous manner as performed for preparing p E1B gal as described above. The same cotransfection and recombination procedure is then followed as described herein to obtain viral gene delivery vectors.

The recombinant viruses thus produced are used as gene delivery tools both in cultured cells and in vivo. For studies of the effectiveness and relative immunogenicity of multiply-deleted vectors, virus particles are produced by growth in the packaging lines described in Example 1 and are purified by CsCl gradient centrifugation. Following titering, virus particles are administered to mice via systemic or local injection or by aerosol delivery to lung. The LacZ reporter gene allows the number and type of cells which are successfully transduced to be evaluated. The duration of transgene expression is evaluated in order to determine the long-term effectiveness of treatment with multiply-deleted recombinant adenoviruses relative to the standard technologies which have been used in clinical trials to date. The immune response to the improved vectors described here is determined by assessing parameters such as inflammation, production of cytotoxic T lymphocytes directed against the vector, and the nature and magnitude of the antibody response directed against viral proteins.

Versions of the vectors which contain therapeutic genes such as CFTR for treatment of cystic fibrosis or tumor suppressor genes for cancer treatment are evaluated in the animal system for safety and efficiency of gene transfer and expression. Following this evaluation, they are used as experimental therapeutic agents in human clinical trials.

B. Retargeting of Adenoviral Gene Delivery Vectors by Producing Viral Particles Containing Different or Altered Fiber Proteins

As the specificity of adenovirus binding to target cells is largely determined by the fiber protein, viral particles that incorporate modified fiber proteins or fiber proteins from different

adenoviral serotypes (pseudotyped vectors) have different specificities. Thus, the expression of the native Ad5 fiber protein in adenovirus packaging cells as described above is also applicable to production of different fiber proteins.

In one aspect of invention, chimeric fiber proteins are produced according to the methods of Stevenson et al., J. Virol., 69:2850-2857 (1995). The authors showed that the determinants for fiber receptor binding activity are located in the head domain of the fiber and that isolated head domain is capable of trimerization and binding to cellular receptors. The head domains of adenovirus type 3 (Ad3) and Ad5 were exchanged in order to produce chimeric fiber proteins. Similar constructs for encoding chimeric fiber proteins for use in the methods of this invention are contemplated. Thus, instead of the using the intact Ad5 fiber-encoding construct prepared in Example 1 as a complementing viral vector in adenoviral packaging cells, the constructs described herein are used to transfect cells along with E4 and/or E1-encoding constructs.

Briefly, full-length Ad5 and Ad3 were amplified from purified adenovirus genomic DNA as a template. The Ad5 and Ad3 nucleotides sequences are available with the respective GenBank Accession Numbers M18369 and M12411. Oligonucleotide primers are designed to amplify the entire coding sequence of the full-length fiber genes, starting from the start codon, ATG, and ending with the termination codon TAA. For cloning purposes, the 5' and 3' primers contain the respective restriction sites BamHI and NotI for cloning into pcDNA plasmid as described in Example 1A. PCR is performed as described above.

The resultant products are then used to construct chimeric fiber constructs by PCR gene overlap extension, as described by Horton et al., BioTechniques, 8:525-535 (1990). The Ad5 fiber tail and shaft regions (5TS; the nucleotide region encoding amino acid residue positions 1 to 403) are connected to the Ad3 fiber head region (3H; the nucleotide region encoding amino acid residue positions 136 to 319) to form the 5TS3H fiber chimera. Conversely, the Ad3 fiber tail and shaft regions (3TS; the nucleotide region encoding amino acid residues positions 1 to 135) are connected to the Ad5 fiber head region (5H; the nucleotide region encoding the amino acid residue positions 404 to 581) to form the 3TSS5H fiber chimera. The fusions are made at the conserved TLWT (SEQ ID NO 21) sequence at the fiber shaft-head junction.

The resultant chimeric fiber PCR products are then digested with BamHI and NotI for separate directional ligation into a similarly digested pcDNA vector. The Ad2 leader sequence is then subcloned into the BamHI as described in Example 1A for preparing an expression vector for subsequent transfection into 211 cells as described above or into the alternative packaging cell systems as previously described. The resultant chimeric fiber construct-containing adenoviral packaging cell lines are then used to complement adenoviral delivery vectors as previously described. Other fiber chimeric constructs are obtained using a similar approach with the various adenovirus serotypes known.

In an alternative embodiment, the methods of this invention contemplate the use of the modified proteins including novel epitopes as described by Michael et al., Gene Therapy, 2:660-668 (1995) and in International Publication WO 95/26412, the disclosures of which are incorporated by reference herein. Both publications describe the construction of a cell-type specific therapeutic viral vector having a new binding specificity incorporated into the virus concurrent with the destruction of the endogenous viral binding specificity. In particular, the authors described the production of an adenoviral vector encoding a gastrin releasing peptide (GRP) at the 3' end of the coding sequence of the Ad5 fiber gene. The resulting fiber-GRP fusion protein was expressed and shown to assemble functional fiber trimers that were correctly transported to the nucleus of HeLa cells following synthesis.

Based on the teachings in the paper and International Publication, similar constructs are contemplated for use in the complementing adenoviral packaging cell systems of this invention for generating new adenoviral gene delivery vectors that are replication-deficient and less immunogenic. Heterologous ligands contemplated for use herein to redirect fiber specificity range from as few as 10 amino acids in size to large globular structures, some of which necessitate the addition of a spacer region so as to reduce or preclude steric hindrance of the heterologous ligand with the fiber or prevent trimerization of the fiber protein. The ligands are inserted at the end or within the linker region. Preferred ligands include those that target specific cell receptors or those that are used for coupling to other moieties such as biotin and avidin. The types of cell signaling as a result of binding by a ligand is dependent upon the specificity of that ligand; i.e., receptor internalization or lack thereof.

A preferred spacer includes a short 12 amino acid peptide linker composed of a series of serines and alanine flanked by a proline residue at each end. One of ordinary skill in the art

is familiar with the preparation of linkers to accomplish sufficient protein presentation and for altering the binding specificity of the fiber protein without compromising the cellular events that follow viral internalization. Moreover, within the context of this invention, preparation of modified fibers having ligands positioned internally within the fiber protein, at the amino terminus and at the carboxy terminus as described below are contemplated for use with the methods described herein.

The preparation of a fiber having a heterologous binding ligand is prepared essentially as described in the above-cited paper. Briefly, for the ligand of choice, site-directed mutagenesis is used to insert the coding sequence for a linker into the *NotI* site at the 3' end of the Ad5 fiber construct in pCLF as prepared in Example 1. The 3' or antisense oligonucleotide encodes a preferred linker sequence of ProSerAlaSerAlaSerAlaSerAlaProGlySer (SEQ ID NO 22) followed by a unique restriction site and two stop codons, respectively, to allow the insertion of a coding sequence for a selected heterologous ligand and to ensure proper translation termination. The 3' end of the antisense oligonucleotide includes sequences that overlap with vector sequence into which the oligonucleotide is inserted via site-directed mutagenesis. Following mutagenesis of the pCLF sequence adding the linker and stop codon sequences, a nucleotide sequence encoding a preselected ligand is obtained, linkers corresponding to the unique restriction site are attached and then the sequence is cloned into linearized corresponding restriction site.

Into the resultant pCLF vector containing a Ad5 fiber gene sequence with 3' nucleotides encoding a linker and a ligand, the Ad2 leader sequence is inserted as previously described. The resultant fiber-ligand construct is then used to transfect 211 or the alternative cell packaging systems previously described to produce complementing viral vector packaging systems for use with the methods of this invention.

In a further embodiment, fiber proteins encoded by fiber genes isolated from different adenoviral serotypes are used intact for transfection into 211 or an alternative cell packaging system as previously described.

A gene encoding the fiber protein of interest is first cloned to create a plasmid analogous to pCLF, and stable cell lines producing the fiber protein are generated as described above for Ad5 fiber. The adenovirus vector described which lacks the fiber gene is then propagated in the cell line producing the fiber protein relevant for the purpose at hand. As the

only fiber gene present is the one in the packaging cells, the adenoviruses produced contain only the fiber protein of interest and therefore have the binding specificity conferred by the complementing protein. Such viral particles are used in studies such as those described above to determine their properties in experimental animal systems.

C. Targeted Gene Delivery Using Viral Vector Particles Lacking Fiber Protein

An alternative mode of entry for adenoviral infection of hematopoietic cells has been described by Huang, et al., *J. Virol.*, 69:2257-2263 (1995) which does not involve the fiber protein-host cell receptor interaction. As infection of most other cell types does require the presence of fiber protein, vector particles which lack fiber may preferentially infect hematopoietic cells, such as monocytes or macrophages.

To produce a fiber-free adenovirus vector particle, a vector lacking the fiber gene as described above in Example 2A but containing a gene of interest for delivery is amplified by growth in cells which do not produce a fiber protein, such as the 211 cells prepared in Example 1, thereby producing large numbers of particles lacking fiber protein. The recovered fiber-free viral particles are then used to deliver the inserted gene of interest following the methods of this invention via targeting mechanisms provided by other regions of the adenoviral vector, i.e., via the native penton base.

Example 3

Deposit of Materials

The following cell lines and plasmids have been deposited on September 25, 1996, with the American Type Culture Collection, 1301 Parklawn Drive, Rockville, MD, USA (ATCC):

<u>Material</u>	<u>ATCC Accession No.</u>
Plasmid pE4/Hygro	97739
Plasmid pCLF	97737
211 Cell Line	CRL-12193
211A Cell Line	CRL-12194

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the

cell lines and plasmids deposited, since the deposited embodiment is intended as a single illustration of one aspect of the invention and any cell lines or plasmid vectors that are functionally equivalent are within the scope of this invention.

The foregoing specification, including the specific embodiments and examples, is intended to be illustrative of the present invention and is not to be taken as limiting. Numerous other variations and modifications can be effected without departing from the true spirit and scope of the present invention.

Example 4

The native fiber protein is a homotrimer (Henry L.J. et al 1994 Characterization of the knob domain of the adenivirus Type 5 fiber protein expressed in *Escherichia coli* J. Virol 68:5239-5246), and trimerization is essential for assembly of the penton/fiber complex (Novelli A et al 1991 Assembly of adenovirus type 2 fiber synthesized in cell-free translation system. J. Biol. Chem 266:9299-9303). To assess the multimeric structure of the recombinant fiber protein produced by the cell lines, cells were labeled with 50 μ Ci/ml [35 S] Translabel (ICN) for two hours at 37 °C, lysed in RIPA buffer, and fiber protein was immunoprecipitated as described (Harlow E et al 1988 Antibodies. Cold Spring Harbour Laboratory, cold Spring Harbour). Immune complexes were collected on Protein A-Sepharose beads (Pierce), extensively washed with RIPA buffer, and incubated at room temperature in 0.1 M triethylamine, pH 11.5 to release bound fiber protein. A portion of the precipitated fiber was electrophoresed on a 8% SDS-PAGE gel under denaturing (1% SDS in loading buffer, samples boiled for 5 minutes) or semi-native (0.1% SDS in loading buffer, samples not heated) conditions.

As seen in Fig. 13, lines 211A, 211B, and 211R, but not the control 293 cells, expressed an immunologically reactive protein which migrated at the predicted molecular weight for trimer (186 kD) under seminative conditions and for monomer (62 kD) under denaturing conditions. The behavior of the precipitated fiber was indistinguishable from that of purified baculovirus-produced recombinant Ad2

fiber { Wickham T et al 1993 Cell 73:309-319} (the 58 kD Ad2 and 62 kD Ad5 fibers have very similar mobilities under these conditions).

To determine whether the fiber-expressing lines could support the growth of a fiber-defective adenovirus, we performed one-step growth experiments using the temperature-sensitive fiber mutant Ad H5ts142 (the gift of Harold Ginsberg). At the restrictive temperature (39.5 °C), this mutant produces an underglycosylated fiber protein which is not incorporated into mature virions { Chee-Sheung C. C et al 1982 J. Virol 42: 932-950 }. This results in the accumulation of non-infectious viral particles. We asked whether the recombinant fiber protein expressed by our cell lines could complement the H5ts142 defect and rescue viral growth.

Cell lines 293, 211A, 211B and 211R (2×10^6 cells/sample) were infected with H5ts142 at 10 pfu/cell. 48 hours later, cells were detached with 25 mM EDTA and virus was harvested by four rapid freeze-thaw cycles. Debris was removed by a 10 minute spin at 1500 x g, and viral titers determined by fluorescent focus assay { Thiel J.F et al 1967 Proc. Soc. Exp. Biol. Med. 125:892-895 } on SW480 cells with a polyclonal anti-penton base Ab { Wickham T et al 1993 Cell 73:309-319}. As shown in Fig. 14, the fiber mutant virus replicated to high titers in 293 cells at 32.5° C (the permissive temperature), but to a much lower extent at the restrictive temperature of 39.5° C. The fiber-producing packaging lines 211A, 211B, or 211R supported virus production at 39.5° C to levels within two- to three-fold of those seen at the permissive temperature in 293 cells, indicating that these cells provided partial complementation of the fiber defect.

Interestingly, virus yields from the fiber-producing cell lines were also somewhat higher than those from 293 cells at 32.5° C (the 'permissive' temperature). This suggests that fiber produced by the ts142 virus may be partially defective even at the permissive temperature. Alternatively, a non-specific increase in adenoviral titer could result when viruses are grown in our packaging cells, by a mechanism not involving fiber complementation. However, we have found that viruses with wild type fiber genes (such as Ad.RSVbgal) replicate to identical levels either in our packaging lines or in 293

cells (data not shown). Taken together, these results demonstrate that the observed increase in H5_{ts}142 growth is due to specific complementation of the fiber mutation.

Even in the fiber-expressing cell lines, the fiber mutant grows to higher titers at 32 °C than at 39.5 °C. This incomplete complementation may be due to the packaging lines' expression of fiber at a level somewhat below that seen in a wild-type infection (Fig. 16). A recent study reported an E4-deleted vector which coincidentally reduced fiber protein expression, resulting in a large reduction in the titer of virus produced { Brough et al 1996 J. Virol. 70: 6497-6501 }. Another possibility is that the defective *ts*142 fiber protein produced at the restrictive temperature might form complexes with some of the wild type protein produced by the cells and prevent its assembly into particles.

Although the fiber proteins of different Ad serotypes differ in the length of their shaft domains and in their receptor-binding knob domains, the N-terminal regions responsible for interaction with the viral penton base are highly conserved { Arnberg N et al 1997 Virology 227:239-244 } (Fig. 15A). This suggests that fibers from many viral serotypes, with their different cell-binding specificities, may be amenable for use in producing gene delivery vectors.

We asked whether the recombinant Ad5 fiber produced by our packaging cells could be incorporated into particles of another adenovirus serotype. Adenovirus type 3 was grown either in fiber-producing cell lines or in 293 cells. Viral particles were purified by two sequential centrifugations (3 h at 111,000 x g) on preformed 15-40% CsCl gradients to remove soluble cellular proteins and then dialyzed extensively against 10 mM Tris-HCl, pH 8.1, 150 mM NaCl, 10% glycerol. Ad5 fiber protein was detected by immunoblotting using the polyclonal anti-fiber serum, followed by detection with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Kirkegaard and Perry Laboratories) and the ECL chemiluminescence substrate (Amersham). The purified Ad3 particles contained Ad5 fiber protein after a single passage through a fiber-expressing cell line but not after passage through 293 cells (Fig. 15B). Previous work has demonstrated that Ad2 fiber is capable of interacting *in vitro* with Ad3 penton base { Fender et al 1997 Nature Biotech. 15:52-56 }, and our result

demonstrates that the type 5 fiber protein produced by the cells is capable of assembling into complete Ad3 particles.

A vector based on Ad5 but containing the gene for the Ad7 fiber protein has been described { Gall J. et al 1996 J. Virol. 70:2116-2123}, as well as Ads containing chimeric fiber genes {Krasnykh V. N et al J. Virol. 70:6839-6846}. Addition of a short peptide linker to the fiber in order to confer binding to a different cellular protein has also been reported { 8188 }. By using packaging technology such as that presented here, Ad vectors equipped with different fiber proteins may be produced simply by growth in cells expressing the fiber of interest, without the time-consuming step of generating a new vector genome for each application.

Replacing or modifying the fiber gene in the vector chromosome would also require that the new fiber protein bind a receptor on the surface of the cells it which it is to be grown. The packaging cell approach will allow the generation of Ad particles containing a fiber which can no longer bind to its host cells, by a single round of growth in cells expressing the desired fiber gene. This will greatly expand the repertoire of fiber proteins which can be incorporated into particles, as well as simplifying the process of retargeting gene delivery vectors.

Finally, a novel fiber-independent pathway of infection has recently been described in hematopoietic cells, in which penton base provides the initial virus-cell interaction by binding to integrin $\alpha_m\beta_2$ {Huang S. et al 1996 J. Virol 70: 4502-4508}. This suggests that viral particles lacking fiber protein may be useful in targeting gene delivery to specific cell types via this pathway.

- 60 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Nemerow, Glen R.
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(ii) TITLE OF INVENTION: PACKAGING CELL LINES, ADENOVIRUS
VECTORS, AND METHODS OF USING SAME

(iii) NUMBER OF SEQUENCES: 20

(iv) CORRESPONDENCE ADDRESS:

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(viii) ATTORNEY/AGENT INFORMATION:

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(C) REFERENCE/DOCKET NUMBER: TSRI 554.0

(ix) TELECOMMUNICATION INFORMATION:

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- 61 -

(B) TELEFAX: (619) 554-6312

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGGTACACAG AATTCAGGAG ACACAACTCC
30

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

- 62 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCCTGGATCC GGGAAAGTTAC GTAACGTGGG AAAAC

35

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGCGGATCCG CG

12

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8710 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CACCTAAATT GTAAGCGTTA ATATTTGTT AAAATTCGCG TTAAATTGGT GTTAAATCAG
60

CTCATTGGT AACCAATAGG CCGAAATCGG CAAAATCCCT TATAAATCAA AAGAATAGAC
120

CGAGATAGGG TTGAGTGTG TTCCAGTTG GAACAAGAGT CCACTATTAA AGAACGTGGA
180

CTCCAACGTC AAAGGGCGAA AAACCGTCTA TCAGGGCGAT GGCCCACCTAC GTGAACCATC
240

ACCTTAATCA AGTTTTTGG GGTCGAGGTG CCGTAAAGCA CTAAATCGGA ACCCTAAAGG
300

GAGCCCCCGA TTTAGAGCTT GACGGGGAAA GCCGGCGAAC GTGGCGAGAA AGGAAGGGAA
360

GAAAGCGAAA GGAGCGGGCG CTAGGGCGCT GGCAAGTGTG GCGGTCACGC TGCGCGTAAC
420

CACCAACACCC GCCGCGCTTA ATGCGCCGCT ACAGGGCGCG TCCCATTGCG CATTCAAGGCT
480

GCGCAACTGT TGGGAAGGGC GATCGGTGCG GGCCTCTTCG CTATTACGCC AGCTGGCGAA
540

AGGGGGATGT GCTGCAAGGC GATTAAGTTG GGTAACGCCA GGGTTTCCC AGTCACGACG
600

TTGTAAAACG ACGGCCAGTG AATTGTAATA CGACTCACTA TAGGGCGAAT TGGGTACCGG
660

GCCCCCCCCTC GAGGTCGACG GTATCGATAA GCTTGATATC GAATTCAAGGA GACACAACCTC
720

CAAGTGCATA CTCTATGTCA TTTTCATGGG ACTGGTCTGG CCACAACTAC ATTAATGAAA
780

TATTTGCCAC ATCCTCTTAC ACTTTTTCAT ACATTCGCCA AGAATAAAGA ATCGTTGTG
840

TTATGTTCA ACGTGTAT TTTCAATTG CAGAAAATT CAAGTCATTT TTCATTCACT
900

AGTATAGCCC CACCACACA TAGCTTATAC AGATCACCGT ACCTTAATCA AACTCACAGA
960

ACCTAGTAT TCAACCTGCC ACCTCCCTCC CAACACACAG AGTACACAGT CCTTCTCCC
1020

CGGCTGGCCT TAAAAAGCAT CATATCATGG GTAACAGACA TATTCTTAGG TGTTATATTC
1080

CACACGGTTT CCTGTCGAGC CAAACGCTCA TCAGTGATAT TAATAAACTC CCCGGGCAGC
1140

TCACTTAAGT TCATGTCGCT GTCCAGCTGC TGAGCCACAG GCTGCTGTCC AACTTGCAGG
1200

TGCTTAACGG GCGGCGAAGG AGAAGTCCAC GCCTACATGG GGGTAGAGTC ATAATCGTGC
1260

ATCAGGATAG GGCGGTGGTG CTGCAGCAGC GCGCGAATAA ACTGCTGCCG CCGCCGCTCC
1320

GTCCTGCAGG AATACAACAT GGCAGTGGTC TCCTCAGCGA TGATTCGCAC CGCCCGCAGC
1380

ATAAGGCGCC TTGTCCTCCG GGCACAGCAG CGCACCCCTGA TCTCACTTAA ATCAGCACAG
1440

TAACTGCAGC ACAGCACCAC AATATTGTTA AAAATCCAC AGTGCAAGGC GCTGTATCCA
1500

AAGCTCATGG CGGGGACCA AGAACCCACG TGGCCATCAT ACCACAAGCG CAGGTAGATT
1560

AAGTGGCGAC CCCTCATAAA CACGCTGGAC ATAAACATTA CCTCTTTGG CATGTTGTAA
1620

TTCACCACCT CCCGGTACCA TATAAACCTC TGATTAACCA TGGGCCATC CACCACCATC
1680

CTAAACCAGC TGGCCAAAAC CTGCCCCCG GCTATACACT GCAGGGAACC GGGACTGGAA
1740

CAATGACAGT GGAGAGCCCA GGACTCGTAA CCATGGATCA TCATGCTCGT CATGATATCA
1800

ATGTTGGCAC AACACAGGCA CACGTGCATA CACTTCCTCA GGATTACAAG CTCCTCCGC
1860

GTTAGAACCA TATCCCAGGG AACAAACCCAT TCCTGAATCA GCGTAAATCC CACACTGCAG
1920

GGAAGACCTC GCACGTAACT CACGTTGTGC ATTGTCAAAG TGTTACATTC GGGCAGCAGC
1980

GGATGATCCT CCAGTATGGT AGCGCGGGTT TCTGTCTCAA AAGGAGGTAG ACGATCCCTA
2040

CTGTACGGAG TGCGCCGAGA CAACCGAGAT CGTGTGGTC GTAGTGTCAATGCCAAATGGA
2100

ACGCCGGACG TAGTCATATT TCCTGAAGCA AAACCAGGTG CGGGCGTGAC AAACAGATCT
2160

GCGTCTCCGG TCTCGCCGCT TAGATCGCTC TGTGTAGTAG TTGTAGTATA TCCACTCTCT
2220

CAAAGCATCC AGGCGCCCCC TGGCTTCGGG TTCTATGTAA ACTCCTTCAT GCGCCGCTGC
2280

CCTGATAACA TCCACCCACCG CAGAATAAGC CACACCCAGC CAACCTACAC ATTCGTTCTG
2340

CGAGTCACAC ACGGGAGGAG CGGGAAAGAGC TGGAAGAACCC ATGTTTTTTT TTTTATTCCA
2400

AAAGATTATC CAAAACCTCA AAATGAAGAT CTATTAAGTG AACGCGCTCC CCTCCGGTGG
2460

CGTGGTCAAA CTCTACAGCC AAAGAACAGA TAATGGCATT TGTAAGATGT TGCACAATGG
2520

CTTCCAAAAG GCAAACGGCC CTCACGTCCA AGTGGACGTA AAGGCTAAAC CCTTCAGGGT
2580

GAATCTCCTC TATAAACATT CCAGCACCTT CAACCATGCC CAAATAATTC TCATCTCGCC
2640

ACCTTCTCAA TATATCTCTA AGCAAATCCC GAATATTAAG TCCGGCCATT GTAAAATCT
2700

GCTCCAGAGC GCCCTCCACC TTCAGCCTCA AGCAGCGAAT CATGATTGCA AAAATTCAAGG
2760

TTCCCTCACAG ACCTGTATAA GATTCAAAAG CGGAACATTA ACAAAAATAC CGCGATCCCG
2820

TAGGTCCCTT CGCAGGGCCA GCTGAACATA ATCGTGCAGG TCTGCACGGG CCAGCGCGGC
2880

CACTTCCCCG CCAGGAACCT TGACAAAAGA ACCCACACTG ATTATGACAC GCATACTCGG
2940

AGCTATGCTA ACCAGCGTAG CCCCCGATGTA AGCTTTGTTG CATGGGCGGC GATATAAAAT
3000

GCAAGGTGCT GCTAAAAAAA TCAGGCAAAG CCTCGCGCAA AAAAGAAAGC ACATCGTAGT
3060

CATGCTCATG CAGATAAAGG CAGGTAAGCT CCGGAACCAC CACAGAAAAA GACACCATT
3120

TTCTCTCAAA CATGTCTGCG GGTTTCTGCA TAAACACAAA ATAAAATAAC AAAAAAAACAT
3180

TTAACACATTA GAAGCCTGTC TTACAACAGG AAAAACAAACC CTTATAAGCA TAAGACGGAC
3240

TACGGCCATG CCGGCGTGAC CGTAAAAAAA CTGGTCACCG TGATTAAAAAA GCACCACCGA
3300

CAGCTCCTCG GTCATGTCCG GAGTCATAAT GTAAGACTCG GTAAACACAT CAGGTTGATT
3360

CATCGGTCAAG TGCTAAAAAG CGACCGAAAT AGCCCCGGGG AATACATACC CGCAGGCGTA
3420

GAGACAACAT TACAGCCCCC ATAGGAGGTA TAACAAAATT AATAGGAGAG AAAACACAT
3480

AAACACCTGA AAAACCTCC TGCCTAGGCA AAATAGCACC CTCCCGCTCC AGAACAAACAT
3540

ACAGCGCTTC ACAGCGGCAG CCTAACAGTC AGCCTTACCA GTAAAAAAGA AAACCTATTA
3600

AAAAAACACC ACTCGACACG GCACCAGCTC AATCAGTCAC AGTGTAAAAA AGGGCCAAGT
3660

GCAGAGCGAG TATATATAGG ACTAAAAAAT GACGTAACGG TTAAAGTCCA CAAAAAACAC
3720

CCAGAAAACC GCACGCGAAC CTACGCCAG AAACGAAAGC CAAAAAACCC ACAACTTCCT
3780

CAAATCGTCA CTTCCGTTTT CCCACGTTAC GTAACTTCCC GGATCCGCGG CATTACAGT
3840

TCTCCGCAAG AATTGATTGG CTCCAATTCT TGGAGTGGTG AATCCGTTAG CGAGGTGCCG
3900

CCGGCTTCCA TTCAGGTCGA GGTGGCCCGG CTCCATGCAC CGCGACGCAA CGCGGGGAGG
3960

CAGACAAGGT ATAGGGCGGC GCCTACAATC CATGCCAACCG CGTTCCATGT GCTGCCGAG
4020

GCGGCATAAA TCGCCGTGAC GATCAGCGGT CCAGTGATCG AAGTTAGGCT GGTAAGAGCC
4080

GCGAGCGATC CTTGAAGCTG TCCCTGATGG TCGTCATCTA CCTGCCTGGA CAGCATGGCC
4140

TGCAACGCGG GCATCCCGAT GCCGCCGGAA GCGAGAAGAA TCATAATGGG GAAGGCCATC
4200

CAGCCTCGCG TCGCGAACGC CAGCAAGACG TAGCCCAGCG CGTCGGCCGC CATGCCCTGC
4260

TTCATCCCCG TGGCCCGTTG CTCGCGTTG CTGGCGGTGT CCCCCGAAGA AATATATTTG
4320

CATGTCTTTA GTTCTATGAT GACACAAACC CCGCCCAGCG TCTTGTCAATT GGCGAATTG
4380

AACACGCAGA TGCAGTCGGG GCGGCGCGGT CCCAGGTCCA CTTCGCATAT TAAGGTGACG
4440

CGTGTGGCCT CGAACACCGA GCGACCCCTGC AGCGACCCGC TTAACAGCGT CAACAGCGTG
4500

CCGCAGATCC CGGGCAATGA GATATGAAAA AGCCTGAACG CACCGCGACG TCTGTCGAGA
4560

AGTTTCTGAT CGAAAAGTTC GACAGCGTCT CCGACCTGAT GCAGCTCTCG GAGGGCGAAG
4620

AATCTCGTGC TTTCAGCTTC GATGTAGGAG GGC GTGGATA TGT CCTGC GG GTAAATAGCT
4680

GCGCCGATGG TTTCTACAAA GATCGTTATG TTTATCGGCA CTTTGCATCG GCC GCGCTCC
4740

CGATTCCGGA AGTGCTTGAC ATTGGGAAAT TCAGCGAGAG CCTGACCTAT TGCATCTCCC
4800

GCCGTGCACA GGGTGTCA CG TTGCAAGACC TGCCTGAAAC CGAACTGCC CGT GTTCTGC
4860

AGCCGGTCGC GGAGGCCATG GATGCGATCG CTGCGGCCGA TCTTAGCCAG ACGAGCGGGT
4920

TCGGCCCATT CGGACCGCAA GGAATCGGTC AATACTAC ATGGCGTGAT TTCATATGCG
4980

CGATTGCTGA TCCCCATGTG TATCACTGGC AAAC TGTGAT GGACGACACC GTCAGTGC GT
5040

CCGTCGCGCA GGCTCTCGAT GAGCTGATGC TTTGGGCCGA GGACTGCC CGAAGTCCGGC
5100

ACCTCGTGC A CGCGGATTTC GGCTCCAACA ATGTCCTGAC GGACAATGGC CGCATAACAG
5160

CGGTCA TTGA CTGGAGCGAG GCGATGTTCG GGGATTCCCA ATACGAGGTC GCCAACATCT
5220

TCTTCTGGAG GCCGTGGTTG GCTTGTATGG AGCAGCAGAC GCGCTACTTC GAGCGGAGGC
5280

ATCCGGAGCT TGCAGGATCG CCGCGGCTCC GGGCGTATAT GCTCCGCATT GGTCTTGACC
5340

AACTCTATCA GAGCTTGGTT GACGGCAATT TCGATGATGC AGCTTGGCG CAGGGTCGAT
5400

GCGACGCAAT CGTCCGATCC GGAGCCGGGA CTGTCGGCG TACACAAATC GCCCGCAGAA
5460

GCGCGGCCGT CTGGACCGAT GGCTGTGTAG AAGTACTCGC CGATAGTGGA AACCGACGCC
5520

CCAGCACTCG TCCGAGGGCA AAGGAATAGG GGAGATGGGG GAGGCTAACT GAAACACGGA
5580

AGGAGACAAT ACCGGAAGGA ACCCGCGCTA TGACGGCAAT AAAAAGACAG AATAAAACGC
5640

ACGGGTGTTG GGTCGTTGT TCATAAACGC GGGGTTCGGT CCCAGGGCTG GCACCTGTG
5700

GATACCCCAC CGAGACCCCA TTGGGGCCAA TACGCCCGCG TTTCTTCCTT TTCCCCACCC
5760

CACCCCCCAA GTTCGGGTGA AGGCCCAGGG CTCGCAGCCA ACGTCGGGGC GGCAAGGCC
5820

GCCATAGCCA CTGGCCCCGT GGGTTAGGGA CGGGGTCCCC CATGGGAAT GGTTTATGGT
5880

TCGTGGGGT TATTATTTG GGC GTTGC GT GGGGTCTGGT CCACGACTGG ACTGAGCAGA
5940

CAGACCCATG GTTTTGGAT GCCCTGGCA TGGACCGCAT GTACTGGCGC GACACGAACA
6000

CCGGGCGTCT GTGGCTGCCA AACACCCCCG ACCCCCCAAA ACCACCGCGC GGATTCTGG
6060

CGCCCCAGTGC CGTCGACCGG TCATGGCTGC GCCCCGACAC CCGCCAACAC CCGCTGACGC
6120

GCCCTGACGG GCTTGTCTGC TCCCGGCATC CGCTTACAGA CAAGCTGTGA CCGTCTCCGG
6180

GAGCTGCATG TGTCAGAGGT TTTCACCGTC ATCACCGAAA CGCGCGAGGC AGCCGGATCA
6240

TAATCAGCCA TACCACATTT GTAGAGGTTT TACTTGCTTT AAAAAACCTC CCCACCTCCC
6300

CCTGAACCTG AAACATAAAA TGAATGCAAT TGTTGTTGTT AACTTGTTA TTGCAGCTTA
6360

TAATGGTTAC AAATAAAGCA ATAGCATCAC AAATTCACA AATAAAGCAT TTTTTCACT
6420

GCATTCTAGT TGTGGTTGT CCAAACATCAT CAATGTATCT TATCATGTCT GGATCCACTA
6480

GTTCTAGAGC GGCGGCCACC GCGGTGGAGC TCCAGCTTTT GTTCCCTTTA GTGAGGGTTA
6540

ATTCGAGCT TGGCGTAATC ATGGTCATAG CTGTTCCCTG TGTGAAATTG TTATCCGCTC
6600

ACAATTCCAC ACAACATACG AGCCGGAAGC ATAAAGTGT AAGCCTGGGG TGCTTAATGA
6660

GTGAGCTAAC TCACATTAAT TGCCTTGCCTC TCACTGCCCG CTTTCCAGTC GGGAAACCTG
6720

TCGTGCCAGC TGCATTAATG AATCGGCCAA CGCGCGGGGA GAGGCGGTTT GCGTATTGGG
6780

CGCTCTTCCG CTTCTCGCT CACTGACTCG CTGCGCTCGG TCGTTGGCT GCGGCGAGCG
6840

GTATCAGCTC ACTCAAAGGC GGTAATACGG TTATCCACAG AATCAGGGGA TAACGCAGGA
6900

AAGAACATGT GAGCAAAAGG CCAGCAAAAG GCCAGGAACC GTAAAAAGGC CGCGTTGCTG
6960

GCCTTTTCC ATAGGCTCCG CCCCCCTGAC GAGCATCACA AAAATCGACG CTCAAGTCAG
7020

AGGTGGCGAA ACCCGACAGG ACTATAAAGA TACCAGGCGT TTCCCCCTGG AAGCTCCCTC
7080

GTGCGCTCTC CTGTTCCGAC CCTGCCGCTT ACCGGATACC TGTCCGCCTT TCTCCCTTCG
7140

GGAAGCGTGG CGCTTTCTCA TAGCTCACGC TGTAGGTATC TCAGTTCGGT GTAGGTCGTT
7200

CGCTCCAAGC TGGGCTGTGT GCACGAACCC CCCGTTCAAGC CCGACCGCTG CGCCTTATCC
7260

GGTAACTATC GTCTTGAGTC CAACCCGGTA AGACACGACT TATGCCACT GGCAGCAGCC
7320

ACTGGTAACA GGATTAGCAG ACCGAGGTAT GTAGGCGGTG CTACAGAGTT CTTGAAGTGG
7380

TGGCCTAACT ACGGCTACAC TAGAAGGACA GTATTTGGTA TCTGCCCTCT GCTGAAGCCA
7440

GTTACCTTCG GAAAAAGAGT TGGTAGCTCT TGATCCGGCA AACAAACCAC CGCTGGTAGC
7500

GGTGGTTTTT TTGTTGCAA GCAGCAGATT ACGCCAGAA AAAAAGGATC TCAAGAAGAT
7560

CCTTTGATCT TTTCTACGGG GTCTGACGCT CAGTGGAACG AAAACTCACG TTAAGGGATT
7620

TTGGTCATGA GATTATCAA AAGGATCTTC ACCTAGATCC TTTTAAATTA AAAATGAAGT
7680

TTTAAATCAA TCTAAAGTAT ATATGAGTAA ACTTGGTCTG ACAGTTACCA ATGCTTAATC
7740

AGTGAGGCAC CTATCTCAGC GATCTGTCTA TTTCGTTCAT CCATAGTTGC CTGACTCCCC
7800

GTCGTGTAGA TAACTACGAT ACGGGAGGGC TTACCATCTG GCCCCAGTGC TGCAATGATA
7860

CCCGAGAGACC CACGCTCACC GGCTCCAGAT TTATCAGCAA TAAACCAGCC AGCCGGAAGG
7920

GCCGAGCGCA GAAGTGGTCC TGCAACTTTA TCCGCCTCCA TCCAGTCTAT TAATTGTTGC
7980

CGGGAAGCTA GAGTAAGTAG TTGCCAGTT AATAGTTTGC GCAACGTTGT TGCCATTGCT
8040

ACAGGCATCG TGGTGTACG CTCGTCGTTT GGTATGGCTT CATTTCAGCTC CGGTTCCCAA
8100

CGATCAAGGC GAGTTACATG ATCCCCCATG TTGTGCAAAA AAGCGGTTAG CTCCCTCGGT
8160

CCTCCGATCG TTGTCAGAAG TAAAGTGGCC GCAGTGTAT CACTCATGGT TATGGCAGCA
8220

CTGCATAATT CTCTTACTGT CATGCCATCC GTAAGATGCT TTTCTGTGAC TGGTGAGTAC
8280

TCAACCAAGT CATTCTGAGA ATAGTGTATG CGGCGACCGA GTTGCTCTTG CCCGGCGTCA
8340

ATACGGGATA ATACCGCGCC ACATAGCAGA ACTTTAAAAG TGCTCATCAT TGGAAAACGT
8400

TCTTCGGGGC GAAAACCTCTC AAGGATCTTA CCGCTGTTGA GATCCAGTTC GATGTAACCC
8460

ACTCGTGCAC CCAACTGATC TTCAGCATCT TTTACTTTCA CCAGCGTTTC TGGGTGAGCA
8520

- 74 -

AAAACAGGAA GGCAAAATGC CGCAAAAAAG GGAATAAGGG CGACACGGAA ATGTTGAATA
8580

CTCATACTCT TCCTTTTCA ATATTATTGA AGCATTATC AGGGTTATTG TCTCATGAGC
8640

GGATACATAT TTGAATGTAT TTAGAAAAAT AAACAAATAG GGGTTCCGCG CACATTTCCC
8700

CGAAAAGTGC
8710

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGGGATCCA AGATGAAGCG CGCAAGACCG
30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CATAACGCGG CCGCTTCTTT ATTCTTGGGC

30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7148 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GACGGGATCGG GAGATCTCCC GATCCCCTAT GGTCGACTCT CAGTACAATC TGCTCTGATG
60

CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT GGAGGGTCGCT GAGTAGTGCG
120

CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC
180

TTAGGGTTAG GCGTTTGCG CTGCTTCGCG ATGTACGGGC CAGATATAACG CGTTGACATT
240

GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGTC ATTAGTTCAT AGCCCATATA
300

TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC
360

CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC
420

ATTGACGTCA ATGGGTGGAC TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT
480

ATCATATGCC AAGTACGCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT
540

ATGCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA
600

TCGCTATTAC CATGGTGATG CGGTTTGCG AGTACATCAA TGGGCGTGGA TAGCGGTTG
660

ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTG TTTTGGCACC
720

AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGCG
780

GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCCA
840

CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG GGAGACCCAA GCTTGGTACC
900

GAGCTCGGAT CCAAGATGAA GCGCGCAAGA CCGTCTGAAG ATACCTTCAA CCCCCGTGTAT
960

CCATATGACA CGGAAACCGG TCCTCCA ACT GTGCCTTTTC TTACTCCTCC CTTTGTATCC
1020

CCCAATGGGT TTCAAGAGAG TCCCCCTGGG GTACTCTCTT TGCGCCTATC CGAACCTCTA
1080

GTTACCTCCA ATGGCATGCT TGCCTCAAA ATGGGCAACG GCCTCTCTC GGACGAGGCC
1140

GGCAACCTTA CCTCCCAAAA TGTAACCACT GTGAGCCCAC CTCTAAAAA AACCAAGTCA
1200

AACATAAACC TGGAAATATC TGCACCCCTC ACAGTTACCT CAGAAGCCCT AACTGTGGCT
1260

GCCGCCGCAC CTCTAATGGT CGCGGGCAAC ACACTCACCA TGCAATCACA GGCCCCGCTA
1320

ACCGTGCACG ACTCCAAACT TAGCATTGCC ACCCAAGGAC CCCTCACAGT GTCAGAAGGA
1380

AAGCTAGCCC TGCAAACATC AGGCCCCCTC ACCACCACCG ATAGCAGTAC CCTTACTATC
1440

ACTGCCTCAC CCCCTCTAAC TACTGCCACT GGTAGCTTGG GCATTGACTT GAAAGAGCCC
1500

ATTTATACAC AAAATGGAAA ACTAGGACTA AAGTACGGGG CTCCTTGCA TGTAACAGAC
1560

GACCTAAACA CTTTGACCGT AGCAACTGGT CCAGGTGTGA CTATTAATAA TACTTCCTTG
1620

CAAACCAAAG TTACTGGAGC CTTGGGTTTT GATTACAAG GCAATATGCA ACTTAATGTA
1680

GCAGGAGGAC TAAGGATTGA TTCTCAAAAC AGACGCCCTTA TACTTGATGT TAGTTATCCG
1740

TTTGATGCTC AAAACCAACT AAATCTAAGA CTAGGACAGG GCCCTCTTT TATAAACTCA
1800

GCCCCACAAC TGGATATTAA CTACAACAAA GGCCTTACT TGTTTACAGC TTCAAACAAT
1860

TCCAAAAGC TTGAGGTTAA CCTAAGCACT GCCAAGGGT TGATGTTGA CGCTACAGCC
1920

ATAGCCATTA ATGCAGGAGA TGGGCTTGAA TTTGGTTCAC CTAATGCACC AAACACAAAT
1980

CCCTCAAAA CAAAAATTGG CCATGGCCTA GAATTGATT CAAACAAGGC TATGGTCCT
2040

AAACTAGGAA CTGGCCTTAG TTTTGACAGC ACAGGTGCCA TTACAGTAGG AAACAAAAAT
2100

AATGATAAGC TAACTTTGTG GACCACACCA GCTCCATCTC CTAACTGTAG ACTAAATGCA
2160

GAGAAAGATG CTAAACTCAC TTTGGTCTTA ACAAAATGTG GCAGTCAAAT ACTTGCTACA
2220

GTTTCAGTTT TGGCTGTTAA AGGCAGTTG GCTCCAATAT CTGGAACAGT TCAAAGTGCT
2280

CATCTTATTA TAAGATTGGA CGAAAATGGA GTGCTACTAA ACAATTCCCT CCTGGACCCA
2340

GAATATTGGA ACTTTAGAAA TGGAGATCTT ACTGAAGGCA CAGCCTATAC AAACGCTGTT
2400

GGATTATGC CTAACCTATC AGCTTATCCA AAATCTCACG GTAAAAGTGC CAAAAGTAAC
2460

ATTGTCAGTC AAGTTACTT AAACGGAGAC AAAACTAAAC CTGTAACACT AACCATTACA
2520

CTAAACGGTA CACAGGAAAC AGGAGACACA ACTCCAAGTG CATACTCTAT GTCATTTCA
2580

TGGGACTGGT CTGGCCACAA CTACATTAAT GAAATATTTG CCACATCCTC TTACACTTT
2640

TCATACATTG CCCAAGAATA AAGAAGCGGC CGCTCGAGCA TGCATCTAGA GGGCCCTATT
2700

CTATAGTGTC ACCTAAATGC TAGAGCTCGC TGATCAGCCT CGACTGTGCC TTCTAGTTGC
2760

CAGCCATCTG TTGTTTGCCTC CTCCCCCGTG CCTTCCTTGA CCCTGGAAGG TGCCACTCCC
2820

ACTGTCCTTT CCTAATAAAA TGAGGAAATT GCATCGCATT GTCTGAGTAG GTGTCATTCT
2880

ATTCTGGGGG GTGGGGTGGG GCAGGACAGC AAGGGGGAGG ATTGGGAAGA CAATAGCAGG
2940

CATGCTGGGG ATGCGGTGGG CTCTATGGCT TCTGAGGCAG AAAGAACCCAG CTGGGGCTCT
3000

AGGGGGTATC CCCACGCGCC CTGTAGCGGC GCATTAAGCG CGGCGGGTGT GGTGGTTACG
3060

CGCAGCGTGA CCGCTACACT TGCCAGCGCC CTAGCGCCCG CTCCCTTCGC TTTCTCCCT
3120

TCCTTTCTCG CCACGTTCGC CGGCTTCCC CGTCAAGCTC TAAATCGGGG CATCCCTTA
3180

GGGTTCCGAT TTAGTGCTTT ACGGCACCTC GACCCCAAAA AACTTGATTA GGGTGATGGT
3240

TCACGTAGTG GGCCATCGCC CTGATAGACG GTTTTCGCC CTTTGACGTT GGAGTCCACG
3300

TTCTTTAATA GTGGACTCTT GTTCCAAACT GGAACAAACAC TCAACCCTAT CTCGGTCTAT
3360

TCTTTGATT TATAAGGGAT TTTGGGGATT TCGGCCTATT GGTTAAAAAA TGAGCTGATT
3420

TAACAAAAAT TTAACCGCAA TTAATTCTGT GGAATGTGTG TCAGTTAGGG TGTGGAAAGT
3480

CCCCAGGCTC CCCAGGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA GTCAGCAACC
3540

AGGTGTGGAA AGTCCCCAGG CTCCCCAGCA GGCAGAAGTA TGCAAAGCAT GCATCTCAAT
3600

TAGTCAGCAA CCATAGTCCC GCCCCTAACT CCGCCCATCC CGCCCTAAC TCCGCCAGT
3660

TCCGCCATT CTCCGCCCA TGGCTGACTA ATTTTTTTA TTTATGCAGA GGCGAGGCC
3720

GCCTCTGCCT CTGAGCTATT CCAGAAGTAG TGAGGAGGCT TTTTTGGAGG CCTAGGCTTT
3780

TGCAAAAGC TCCCAGGAGC TTGTATATCC ATTTTCGGAT CTGATCAAGA GACAGGATGA
3840

GGATCGTTTC GCATGATTGA ACAAGATGGA TTGCACGCAG GTTCTCCGGC CGCTTGGGTG
3900

GAGAGGCTAT TCGGCTATGA CTGGGCACAA CAGACAATCG GCTGCTCTGA TGCCGCCGTG
3960

TTCCGGCTGT CAGCGCAGGG GCGCCCGGTT CTTTTGTCA AGACCGACCT GTCCGGTGCC
4020

CTGAATGAAC TGCAGGACGA GGCAGCGCGG CTATCGTGGC TGGCCACGAC GGGCGTTCCCT
4080

TGCCAGCTG TGCTCGACGT TGTCACTGAA GCGGGAAAGGG ACTGGCTGCT ATTGGCGAA
4140

GTGCCGGGGC AGGATCTCCT GTCATCTCAC CTTGCTCCTG CCGAGAAAGT ATCCATCATG
4200

GCTGATGCAA TGCAGGGCT GCATACGCTT GATCCGGCTA CCTGCCATT CGACCACCAA
4260

GCGAACATC GCATCGAGCG AGCACGTACT CGGATGGAAG CCGGTCTTGT CGATCAGGAT
4320

GATCTGGACG AAGAGCATCA GGGGCTCGCG CCAGCCGAAC TGTCGCCAG GCTCAAGGCG
4380

CGCATGCCCG ACGCGAGGA TCTCGTCGTG ACCCATGGCG ATGCCTGCTT GCCGAATATC
4440

ATGGTGGAAA ATGGCCGCTT TTCTGGATTTC ATCGACTGTG GCCGGCTGGG TGTGGCGGAC
4500

CGCTATCAGG ACATAGCGTT GGCTACCCGT GATATTGCTG AAGAGCTTGG CGGCGAATGG
4560

GCTGACCGCT TCCTCGTGCT TTACGGTATC GCCGCTCCCG ATTTCGAGCG CATCGCCTTC
4620

TATCGCCTTC TTGACGAGTT CTTCTGAGCG GGACTCTGGG GTTCGAAATG ACCGACCAAG
4680

CGACGCCAA CCTGCCATCA CGAGATTCG ATTCCACCGC CGCCTTCTAT GAAAGGTTGG
4740

GCTTCGGAAT CGTTTCCGG GACGCCGGCT GGATGATCCT CCAGCGCGGG GATCTCATGC
4800

TGGAGTTCTT CGCCCACCCC AACTTGTAA TTGCAGCTTA TAATGGTTAC AAATAAAGCA
4860

ATAGCATCAC AAATTCACA AATAAAGCAT TTTTTCACT GCATTCTAGT TGTGGTTGT
4920

CCAAACTCAT CAATGTATCT TATCATGTCT GTATAACCGTC GACCTCTAGC TAGAGCTTGG
4980

CGTAATCATG GTCATAGCTG TTTCTGTGT GAAATTGTTA TCCGCTCACA ATTCCACACA
5040

ACATACGAGC CGGAAGCATA AAGTGTAAAG CCTGGGGTGC CTAATGAGTG AGCTAACTCA
5100

CATTAATTGC GTTGCCTCA CTGCCCGCTT TCCAGTCGGG AACCTGTCG TGCCAGCTGC
5160

ATTAATGAAT CGGCCAACGC CGGGGGAGAG GCGGTTTGC GATTGGGC GCTTCCGCTT
5220

CCTCGCTCAC TGACTCGCTG CGCTCGGTG TTCGGCTGCG GCGAGCGGT ATCAGCTCACT
5280

CAAAGGCGGT AATACGGTTA TCCACAGAAT CAGGGATAA CGCAGGAAAG AACATGTGAG
5340

CAAAAGGCCA GCAAAAGGCC AGGAACCGTA AAAAGGCC GTTGCTGGCG TTTTTCCATA
5400

GGCTCCGCC CCCTGACGAG CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC
5460

CGACAGGACT ATAAAGATAAC CAGGCCTTTC CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG
5520

TTCCGACCCCT GCCGCTTACC GGATACCTGT CCGCCTTCT CCCTTCGGGA AGCGTGGCGC
5580

TTTCTCAATG CTCACGCTGT AGGTATCTCA GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG
5640

GCTGTGTGCA CGAACCCCCC GTTCAGCCCC ACCGCTGCGC CTTATCCGGT AACTATCGTC
5700

TTGAGTCCAA CCCGGTAAGA CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA
5760

TTAGCAGAGC GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG CCTAACTACG
5820

GCTACACTAG AAGGACAGTA TTTGGTATCT GCGCTCTGCT GAAGCCAGTT ACCTTCGGAA
5880

AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC AAACCACCGC TGGTAGCGGT GGTTTTTTG
5940

TTTGCAAGCA GCAGATTACG CGCAGAAAAA AAGGATCTCA AGAAGATCCT TTGATCTTT
6000

CTACGGGGTC TGACGCTCAG TGGAACGAAA ACTCACGTTA AGGGATTTG GTCATGAGAT
6060

TATCAAAAAG GATCTTCACC TAGATCCTTT TAAATTAAAA ATGAAGTTT AAATCAATCT
6120

AAAGTATATA TGAGTAAACT TGGTCTGACA GTTACCAATG CTTAACAGT GAGGCACCTA
6180

TCTCAGCGAT CTGTCTATTT CGTTCATCCA TAGTTGCCTG ACTCCCCGTC GTGTAGATAA
6240

CTACGATAACG GGAGGGCTTA CCATCTGGCC CCAGTGCTGC AATGATAACCG CGAGACCCAC
6300

GCTCACCGGC TCCAGATTAA TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA
6360

GTGGTCCTGC AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG GAAGCTAGAG
6420

TAAGTAGTTC GCCAGTTAAT AGTTTGCAGCA ACGTTGTTGC CATTGCTACA GGCATCGTGG
6480

TGTCACGCTC GTCGTTGGT ATGGCTTCAT TCAGCTCCGG TTCCCAACGA TCAAGGGCAG
6540

TTACATGATC CCCCATGTTG TGCAAAAAAG CGGTTAGCTC CTTCGGTCTT CCGATCGTTG
6600

TCAGAAGTAA GTTGGCCGCA GTGTTATCAC TCATGGTTAT GGCAGCACTG CATAATTCTC
6660

TTACTGTCAT GCCATCCGTA AGATGTTTT CTGTGACTGG TGAGTACTCA ACCAAGTCAT
6720

TCTGAGAATA GTGTATGCGG CGACCGAGTT GCTCTGCCG GGCAGCAATA CGGGATAATA
6780

CCCGGCCACA TAGCAGAACT TAAAGTGC TCATCATTGG AAAACGTTCT TCGGGGCGAA
6840

AACTCTCAAG GATCTTACCG CTGTTGAGAT CCAGTTGAT GTAACCCACT CGTGCACCCA
6900

ACTGATCTTC AGCATCTTT ACTTCACCA GCGTTCTGG GTGAGCAAAA ACAGGAAGGC
6960

AAAATGCCGC AAAAAGGGA ATAAGGGCGA CACGGAAATG TTGAATACTC ATACTCTTCC
7020

TTTTCAATA TTATTGAAGC ATTTATCAGG GTTATTGTCT CATGAGCGGA TACATATTTG
7080

AATGTATTTA GAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC
7140

CTGACGTC
7148

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7469 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GACGGATCGG GAGATCTCCC GATCCCCTAT GGTCGACTCT CAGTACAATC TGCTCTGATG
60

CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTTGTGTGTT GGAGGTCGCT GAGTAGTGCG
120

CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC
180

TTAGGGTTAG GCGTTTGCG CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT
240

GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGTC ATTAGTTCAT AGCCCATATA
300

TGGAGTTCCG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC
360

CCCCCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC
420

ATTGACGTCA ATGGGTGGAC TATTTACGGT AAACGTGCCA CTTGGCAGTA CATCAAGTGT
480

ATCATATGCC AAGTACGCCA CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT
540

ATGCCAGTA CATGACCTTA TGGGACTTTC CTACTGGCA GTACATCTAC GTATTAGTCA
600

TCGCTATTAC CATGGTGATG CGGTTTGCG AGTACATCAA TGGGCGTGGAA TAGCGGTTG
660

ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTG TTTTGGCACC
720

AAAATCAACG GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGCG
780

GTAGGCGTGT ACGGTGGAG GTCTATATAA GCAGAGCTCT CTGGCTAACT AGAGAACCA
840

CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG GGAGACCCAA GCTTGGTACC
900

GAGCTCGGAT CTGAATTGCA GCTCGCTGTT GGGCTCGCGG TTGAGGACAA ACTCTTCGCG
960

GTCTTCCAG TACTCTTGGAA TCGGAAACCC GTCGGCCTCC GAACGGTACT CCGCCACCGA
1020

GGGACCTGAG CGAGTCGCA TCGACCGGAT CGGAAAACCT CTCGAGAAAG GCGTCTAAC
1080

AGTCACAGTC GCAAGGTAGG CTGAGCACCG TGGCGGGCGG CAGCGGGTGG CGGTGGGGT
1140

TGTTTCTGGC GGAGGTGCTG CTGATGATGT AATTAAAGTA GGCGGTCTTG AGACGGCGGA
1200

TGGTCGAGGT GAGGTGTGGC AGGCTTGAGA TCCAAGATGA AGCGCGCAAG ACCGTCTGAA
1260

GATACCTTCA ACCCCGTGTA TCCATATGAC ACGGAAACCG GTCCTCCAAC TGTGCCTTTT
1320

CTTACTCCTC CCTTTGTATC CCCCAATGGG TTTCAAGAGA GTCCCCCTGG GGTACTCTCT
1380

TTGCGCCTAT CCGAACCTCT AGTTACCTCC AATGGCATGC TTGCGCTCAA AATGGGCAAC
1440

GGCCTCTCTC TGGACGAGGC CGGCAACCTT ACCTCCAAA ATGTAACCAC TGTGAGGCCA
1500

CCTCTCAAAA AAACCAAGTC AAACATAAAC CTGGAAATAT CTGCACCCCT CACAGTTACC
1560

TCAGAAGCCC TAACTGTGGC TGCGCCGCA CCTCTAATGG TCGCGGGCAA CACACTCACC
1620

ATGCAATCAC AGGCCCCGCT AACCGTGCAC GACTCCAAAC TTAGCATTGC CACCCAAGGA
1680

CCCCTCACAG TGTCAGAAGG AAAGCTAGCC CTGCAAACAT CAGGCCCCCT CACCACCACC
1740

GATAGCAGTA CCCTTACTAT CACTGCCTCA CCCCCCTCTAA CTACTGCCAC TGGTAGCTTG
1800

GGCATTGACT TGAAAGAGCC CATTATACA CAAAATGGAA AACTAGGACT AAAGTACGGG
1860

GCTCCTTGCG ATGTAACAGA CGACCTAAAC ACTTTGACCG TAGCAACTGG TCCAGGTGTG
1920

ACTATTAATA ATACTTCCTT GCAAACCTAA GTTACTGGAG CCTTGGGTTT TGATTCACAA
1980

GGCAATATGC AACTTAATGT ACCAGGAGGA CTAAGGATTG ATTCTCAAAA CAGACGCCTT
2040

ATACTTGATG TTAGTTATCC GTTTGATGCT CAAAACCAAC TAAATCTAAG ACTAGGACAG
2100

GGCCCTCTTT TTATAAACTC AGCCCACAAAC TTGGATATTA ACTACAACAA AGGCCTTTAC
2160

TTGTTTACAG CTTCAACAA TTCCAAAAG CTTGAGGTTA ACCTAAGCAC TGCCAAGGGG
2220

TTGATGTTG ACGCTACAGC CATAGCCATT AATGCAGGAG ATGGGCTTGA ATTTGGTTCA
2280

CCTAATGCAC CAAACACAAA TCCCCTCAAA ACAAAAATTG GCCATGGCCT AGAATTTGAT
2340

TCAAACAAGG CTATGGTTCC TAAACTAGGA ACTGGCCTTA GTTTGACAG CACAGGTGCC
2400

ATTACAGTAG GAAACAAAAA TAATGATAAG CTAACTTGT GGACCACACC AGCTCCATCT
2460

CCTAACTGTA GACTAAATGC AGAGAAAGAT GCTAAACTCA CTTTGGTCTT AACAAAATGT
2520

GGCAGTCAAA TACTTGCTAC AGTTTCAGTT TTGGCTGTTA AAGGCAGTTT GGCTCCAATA
2580

TCTGGAACAG TTCAAAGTGC TCATCTTATT ATAAGATTTG ACGAAAATGG AGTGCTACTA
2640

AACAATTCCCT TCCTGGACCC AGAATATTGG AACTTTAGAA ATGGAGATCT TACTGAAGGC
2700

ACAGCCTATA CAAACGCTGT TGGATTTATG CCTAACCTAT CAGCTTATCC AAAATCTCAC
2760

GGTAAAATG CCAAAAGTAA CATTGTCAGT CAAGTTTACT TAAACGGAGA CAAAATCAA
2820

CCTGTAACAC TAACCATTAC ACTAAACGGT ACACAGGAAA CAGGAGACAC AACTCCAAGT
2880

GCATACTCTA TGTCATTTTC ATGGGACTGG TCTGGCCACA ACTACATTAA TGAAATATTT
2940

GCCACATCCT CTTACACTTT TTCATACATT GCCCAAGAAT AAAGAAGCGG CCGCTCGAGC
3000

ATGCATCTAG AGGGCCCTAT TCTATAGTGT CACCTAAATG CTAGAGCTCG CTGATCAGCC
3060

TCGACTGTGC CTTCTAGTTG CCAGCCATCT GTTGTGCGC CCTCCCCCGT GCCTTCCTTG
3120

ACCCCTGGAAG GTGCCACTCC CACTGTCCTT TCCTAATAAA ATGAGGAAAT TGCATCGCAT
3180

TGCTGAGTA GGTGTCATTC TATTCTGGGG GGTGGGGTGG GGCAGGACAG CAAGGGGGAG
3240

GATTGGGAAG ACAATAGCAG GCATGCTGGG GATGCCGTGG GCTCTATGGC TTCTGAGGCG
3300

GAAAGAACCA GCTGGGGCTC TAGGGGGTAT CCCCACGCGC CCTGTAGCGG CGCATTAAAGC
3360

GCAGCGGGGTG TGGTGGTTAC GCCCAGCGTG ACCGCTACAC TTGCCAGCGC CCTAGCGCCC
3420

GCTCCTTTCG CTTTCTTCCC TTCCCTTCTC GCCACGTTCG CCGGCTTCC CCGTCAAGCT
3480

CTAAATCGGG GCATCCCTTT AGGGTTCCGA TTTAGTGCTT TACGGCACCT CGACCCAAA
3540

AAACTTGATT AGGGTGATGG TTCACGTAGT GGGCCATCGC CCTGATAGAC GGTTTTTCGC
3600

CCTTGACGT TGGAGTCCAC GTTCTTTAAT AGTGGACTCT TGTTCAAAC TGGAACAACA
3660

CTCAACCCTA TCTCGGTCTA TTCTTTGAT TTATAAGGGA TTTTGGGAT TTCGGCCTAT
3720

TGGTTAAAAA ATGAGCTGAT TTAACAAAAA TTTAACGCGA ATTAATTCTG TGGAATGTGT
3780

GTCAGTTAGG GTGTGGAAAG TCCCCAGGCT CCCCAGGCAG GCAGAAGTAT GCAAAGCATG
3840

CATCTCAATT AGTCAGCAAC CAGGTGTGGA AAGTCCCCAG GCTCCCCAGC AGGCAGAAGT
3900

ATGCAAAGCA TGCATCTCAA TTAGTCAGCA ACCATAGTCC CGCCCCCTAAC TCCGCCATC
3960

CCGCCCCCTAA CTCCGCCAG TTCCGCCAT TCTCCGCCAG ATGGCTGACT AATTTTTTTT
4020

ATTTATGCAG AGGCCGAGGC CGCCTCTGCC TCTGAGCTAT TCCAGAAGTA GTGAGGAGGC
4080

TTTTTGAG GCCTAGGCTT TTGCAAAAG CTCCCGGGAG CTTGTATATC CATTTCGGA
4140

TCTGATCAAG AGACAGGATG AGGATCGTT CGCATGATTG AACAAAGATGG ATTGCACGCA
4200

GGTTCTCCGG CCGCTTGGGT GGAGAGGCTA TTCCGGCTATG ACTGGGCACA ACAGACAATC
4260

GGCTGCTCTG ATGCCGCCGT GTTCCGGCTG TCAGCGCAGG GGCGCCCGGT TCTTTTGTC
4320

AAGACCGACC TGTCCGGTGC CCTGAATGAA CTGCAGGACG AGGCAGCGCG GCTATCGTGG
4380

CTGGCCACGA CGGGCGTTCC TTGCGCAGCT GTGCTCGACG TTGTCACTGA AGCGGGAAGG
4440

GACTGGCTGC TATTGGGCGA AGTGCCGGGG CAGGATCTCC TGTCATCTCA CCTTGCTCCT
4500

GCCGAGAAAG TATCCATCAT GGCTGATGCA ATGCGCGGC TGCATAACGCT TGATCCGGCT
4560

ACCTGCCCAT TCGACCACCA AGCGAAACAT CGCATCGAGC GAGCACGTAC TCGGATGGAA
4620

GCCGGTCTTG TCGATCAGGA TGATCTGGAC GAAGAGCATC AGGGGCTCGC GCCAGCCGAA
4680

CTGTTGCCA GGCTCAAGGC GCGCATGCC GACGGCGAGG ATCTCGTCGT GACCCATGGC
4740

GATGCCTGCT TGCCGAATAT CATGGTGGAA AATGGCCGCT TTTCTGGATT CATCGACTGT
4800

GGCCGGCTGG GTGTGGCGGA CCGCTATCAG GACATAGCGT TGGCTACCCG TGATATTGCT
4860

GAAGAGCTTG GCGGCGAATG GGCTGACCGC TTCTCGTGC TTTACGGTAT CGCCGCTCCC
4920

GATTCGCAGC GCATGCCCTT CTATGCCCTT CTTGACGAGT TCTTCTGAGC GGGACTCTGG
4980

GGTCGAAAT GACCGACCAA GCGACGCCCA ACCTGCCATC ACGAGATTC GATTCCACCG
5040

CCGCCTTCTA TGAAAGCTTG GGCTTCGGAA TCGTTTCCG GGACGCCGGC TGGATGATCC
5100

TCCAGCGCGG GGATCTCATG CTGGAGTTCT TCGCCCACCC CAACTTGTTC ATTGCAGCTT
5160

ATAATGGTTA CAAATAAACG AATAGCATCA CAAATTCAC AAATAAAAGCA TTTTTTTCAC
5220

TGCATTCTAG TTGTGGTTTG TCCAAACTCA TCAATGTATC TTATCATGTC TGTATACCGT
5280

CGACCTCTAG CTAGAGCTTG GCGTAATCAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT
5340

ATCCGCTCAC AATTCCACAC AACATACGAG CCGGAAGCAT AAAGTGTAAA GCCTGGGTG
5400

CCTAATGAGT GAGCTAACTC ACATTAATTG CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG
5460

GAAACCTGTC GTGCCAGCTG CATTAATGAA TCGGCCAACG CGCGGGGAGA GGCGGTTTGC
5520

GTATTGGGCG CTCTTCCGCT TCCTCGCTCA CTGACTCGCT GCGCTCGGTC GTTCGGCTGC
5580

GGCGAGCGGT ATCAGCTCAC TCAAAGGCGG TAATACGGTT ATCCACAGAA TCAGGGATA
5640

ACGCAGGAAA GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCCG
5700

CGTTGCTGGC GTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA AATCGACGCT
5760

CAAGTCAGAG GTGGCGAAC CCGACAGGAC TATAAAGATA CCAGGCCTTT CCCCCCTGGAA
5820

GCTCCCTCGT GCGCTCTCCT GTTCCGACCC TGCCGCTTAC CGGATACCTG TCCGCCTTTC
5880

TCCCTTCGGG AAGCGTGGCG CTTTCTCAAT GCTCACGCTG TAGGTATCTC AGTCGGTGT
5940

AGGTCGTTCG CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG
6000

CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA TCGCCACTGG
6060

CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT AGGCGGTGCT ACAGAGTTCT
6120

TGAAGTGGTG GCCTAACTAC GGCTACACTA GAAGGACAGT ATTTGGTATC TGCGCTCTGC
6180

TGAAGCCAGT TACCTTCGGA AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG
6240

CTGGTAGCGG TGGTTTTTTT GTTGCAAGC ACCAGATTAC GCGCAGAAAA AAAGGATCTC
6300

AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA AACTCACGTT
6360

AAGGGATTTT GGTCATGAGA TTATCAAAAAA GGATCTTCAC CTAGATCCTT TTAAATTAAA
6420

AATGAAGTTT TAAATCAATC TAAAGTATAT ATGAGTAAAC TTGGTCTGAC AGTTACCAAT
6480

GCTTAATCAG TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT
6540

GACTCCCCGT CGTGTAGATA ACTACGATAAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG
6600

CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA AACCAAGCCAG
6660

CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTATC CGCCTCCATC CAGTCTATTA
6720

ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA TAGTTGCCG AACGTTGTTG
6780

CCATTGCTAC AGGCATCGTG GTGTCACGCT CGTCGTTGG TATGGCTTCA TTCAGCTCCG
6840

GTTCCCAACG ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGAAAAAAA GCGGTTAGCT
6900

CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTATCA CTCATGGTTA
6960

TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT AAGATGCTTT TCTGTGACTG
7020

GTGAGTACTC AACCAAGTCA TTCTGAGAAT AGTGTATGCG GCGACCGAGT TGCTCTTGCC
7080

CGGCGTCAAT ACGGGATAAT ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG
7140

GAAAACGTTT TTGGGGCGA AACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA
7200

TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTT TACTTTCACC AGCGTTCTG
7260

GGTGAGCAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT
7320

GTTGAATACT CATACTCTTC CTTTTCAAT ATTATTGAAG CATTATCAG GGTTATTGTC
7380

TCATGAGCGG ATACATATTT GAATGTATTT AGAAAAATAA ACAAAATAGGG GTTCCGCGCA
7440

CATTTCCCCG AAAAGTGCCA CCTGACGTC
7469

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGCTTAAGCG GCCGCGAAGG AGAAGTCC
28

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCGAGCTAGC GACTGAAAT GAG

23

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCTCTCGAGA GACAGCAAGA CAC

23

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11152 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AAGCTTGGGC AGAAATGGTT GAACTCCCGA GAGTGTCCCTA CACCTAGGGG AGAAGCAGCC
60

AAGGGGTTGT TTCCCCACCAA GGACGACCCG TCTGCGCACA AACGGATGAG CCCATCAGAC
120

AAAGACATAT TCATTCTCTG CTGCAAACCTT GGCATAGCTC TGCTTTGCCT GGGGCTATTG
180

GGGGAAGTTG CGGTTCGTGC TCGCAGGGCT CTCACCC TTG ACTCTTTAA TAGCTCTTCT
240

GTGCAAGATT ACAATCTAAA CAATTGGAG AACTCGACCT TCCTCCTGAG GCAAGGACCA
300

CAGCCAACCTT CCTCTTACAA GCCGCATCGA TTTTGTCCCTT CAGAAATAGA AATAAGAATG
360

CTTGCTAAAA ATTATATTTT TACCAATAAG ACCAATCCAA TAGGTAGATT ATTAGTTACT
420

ATGTTAAGAA ATGAATCATT ATCTTTAGT ACTATTTTA CTCAAATTCA GAAGTTAGAA
480

ATGGGAATAG AAAATAGAAA GAGACGCTCA ACCTCAATTG AAGAACAGGT GCAAGGACTA
540
TTGACCACAG GCCTAGAAGT AAAAAAGGGA AAAAAGAGTG TTTTGTCAA AATAGGAGAC
600

AGGTGGTGGC AACCAAGGGAC TTATAGGGGA CCTTACATCT ACAGACCAAC AGATGCC
660

TTACCATATA CAGGAAGATA TGACTTAAAT TGGGATAGGT GGGTTACAGT CAATGGCTAT
720

AAAGTGTAT ATAGATCCCT CCCTTTCGT GAAAGACTCG CCAGAGCTAG ACCTCCTTGG
780

TGTATGTTGT CTCAAGAAGA AAAAGACGAC ATGAAACAAC AGGTACATGA TTATATTTAT
840

CTAGGAACAG GAATGCACTT TTGGGAAAG ATTTCCATA CCAAGGAGGG GACAGTGGCT
900

GGACTAATAG AACATTATTC TGCAAAACT CATGGCATGA GTTATTATGA ATAGCCTTA
960

TTGGCCCAAC CTTGCGGTTC CCAGGGCTTA AGTAAGTTT TGGTACAAA CTGTTCTTAA
1020

AACGAGGATG TGAGACAAGT GGTTCCCTGA CTTGGTTGG TATCAAAGGT TCTGATCTGA
1080

GCTCTGAGTG TTCTATTTTC CTATGTTCTT TTGGAATTAA TCCAAATCTT ATGTAAATGC
1140

TTATGTAAAC CAAGATATAA AAGAGTGCTG ATTTTTGAG TAAACTTGCA ACAGTCCTAA
1200

CATTCACCTC TTGTGTGTTT GTGTCTGTTC GCCATCCCGT CTCCGCTCGT CACTTATCCT
1260

TCACTTCCA GAGGGTCCCC CCGCAGACCC CGGCGACCCCT CAGGTCGGCC GACTGCGGCA
1320

GCTGGCGCCC GAACAGGGAC CCTCGGATAA GTGACCCCTTG TCTCTATTC TACTATTTGG
1380

TGTTTGTCTT GTATTGTCTC TTTCTTGTCT GGCTATCATC ACAAGAGCGG AACGGACTCA
1440

CCATAGGGAC CAAGCTAGCG ACTGAAAATG AGACATATTA TCTGCCACGG AGGTGTTATT
1500

ACCGAAGAAA TGGCCGCCAG TCTTTGGAC CAGCTGATCG AAGAGGTACT GGCTGATAAT
1560

CTTCCACCTC CTAGCCATT TGAACCACCT ACCCTTCACG AACTGTATGA TTTAGACGTG
1620

ACGGCCCCCG AAGATCCCAA CGAGGAGGCG GTTTCGCAGA TTTTTCCCGA CTCTGTAATG
1680

TTGGCGGTGC AGGAAGGGAT TGACTTACTC ACTTTCCGC CGGCGCCCGG TTCTCCGGAG
1740

CCGCCTCACC TTTCCCGCA GCCCGAGCAG CCGGAGCAGA GAGCCTTGGG TCCGGTTTCT
1800

ATGCCAAACC TTGTACCGGA GGTGATCGAT CTTACCTGCC ACGAGGCTGG CTTTCCACCC
1860

AGTGACGACG AGGATGAAGA GGGTGAGGAG TTTGTGTTAG ATTATGTGGA GCACCCCGGG
1920

CACGGTTGCA GGTCTTGTCA TTATCACCGG AGGAATACGG GGGACCCAGA TATTATGTGT
1980

TCGCTTGCT ATATGAGGAC CTGTGGCATG TTTGTCTACA GTAAGTAAA ATTATGGGCA
2040

GTGGGTGATA GAGTGGTGGG TTTGGTGTGG TAATTTTTT TTTAATTTT ACAGTTTGT
2100

GGTTTAAAGA ATTTGTATT GTGATTTTT TAAAAGGTCC TGTGTCTGAA CCTGAGCCTG
2160

AGCCCGAGCC AGAACCGGAG CCTGCAAGAC CTACCCGCCG TCCTAAAATG GCGCCTGCTA
2220

TCCTGAGACG CCCGACATCA CCTGTGTCTA GAGAATGCAA TAGTAGTACG GATAGCTGTG
2280

- 100 -

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2340

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2400

TTAACGAGCC TGGGCAACCT TTGGACTTGA GCTGTAAACG CCCCAGGCCA TAAGGTGTAA
2460

ACCTGTGATT GCGTGTGTGG TTAACGCCTT TGTTGCTGA ATGAGTTGAT GTAAGTTAA
2520

TAAAGGGTGA GATAATGTTT AACTTGCATG GCGTGTAAA TGGGGCGGGG CTTAAAGGGT
2580

ATATAATGCC CCGTGGGCTA ATCTTGGTTA CATCTGACCT CATGGAGGCT TGGGAGTGT
2640

TGGAAGATTT TTCTGCTGTG CGTAACCTGC TGGAACAGAG CTCTAACAGT ACCTCTTGGT
2700

TTTGGAGGTT TCTGTGGGC TCATCCCAGG CAAAGTTAGT CTGCAGAATT AAGGAGGATT
2760

ACAAGTGGGA ATTTGAAGAG CTTTGAAAT CCTGTGGTGA GCTGTTGAT TCTTGAAATC
2820

TGGGTACCCA GGCGCTTTTC CAAGAGAAGG TCATCAAGAC TTTGGATTTT TCCACACCGG
2880

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2940

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3000

TGAGACACAA GAATCGCCTG CTACTGTTGT CTTCCGTCCG CCCGGCGATA ATACCGACGG
3060

AGGAGCAGCA GCAGCAGCAG GAGGAAGCCA GGCGCGCG GCAGGAGCAG AGCCCATGGA
3120

ACCCGAGAGC CGGCCTGGAC CCTCGGGAAT GAATGTTGTA CAGGTGGCTG AACTGTATCC
3180

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3240

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3300

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3360

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3420

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3480

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3540

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3600

GCCGGGGGTG CTTGGCATGG ACGGGGTGGT TATTATGAAT GTAAGGTTA CTGGCCCCAA
3660

TTTAGCGGT ACGGTTTCC TGGCCAATAC CAACCTTATC CTACACGGTG TAAGCTTCTA
3720

TGGGTTAAC AATACCTGTG TGGAAAGCCTG GACCGATGTA AGGGTTGGG GCTGTGCCTT
3780

TTACTGCTGC TGGAAAGGGGG TGGTGTGTCG CCCCAAAAGC AGGGCTTCAA TTAAGAAATG
3840

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3900

TGTGGCCTCC GACTGTGGTT GCTTCATGCT AGTAAAAGC GTGGCTGTGA TTAAGCATAA
3960

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4020

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4080

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4140

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4200

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4260

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4380

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4440

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4500

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4560

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4620

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4680

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4740

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4800

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4860

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4920

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4980

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5040

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5100

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5160

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5220

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5280

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5340

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5400

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5520

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5580

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5640

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5700

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5760

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5820

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5880

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5940

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6000

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6060

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6120

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6180

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6240

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6300

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6360

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6420

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6480

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6540

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6600

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6660

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6720

CTTGTCACC ATCTCGCAA AACCGGCTGG TCGTCCGCTG GTTGATGACT ATGTTGTTGA
6780

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6840

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6900

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6960

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7020

GCCGCTTTAA TCACGGCGCA CAACCGCCTG TGCAGTCGGC CCTTGATGGT AAAACCATCC
7080

CTCACTGGTA TCGCATGATT AACCGTCTGA TGTGGATCTG GCGCGGCATT GACCCACGCG
7140

AAATCCTCGA CGTCCAGGCA CGTATTGTGA TGAGCGATGC CGAACGTACC GACGATGATT
7200

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7260

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7320

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7380

ATTGTTTGTG TATTTAGAT TCCAACCTAT GGAACGTGATG AATGGGAGCA GTGGTGGAAAT
7440

GCCTTTAATG AGGAAAACCT GTTTGCTCA GAAGAAATGC CATCTAGTGA TGATGAGGCT
7500

ACTGCTGACT CTCAACATTC TACTCCTCCA AAAAAGAAGA GAAAGGTAGA AGACCCCAAG
7560

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7620

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7680

GAAAAATATT CTGTAACCTT TATAAGTAGG CATAACAGTT ATAATCATAA CATACTGTTT
7740

TTTCTTACTC CACACAGGCA TAGAGTGTCT GCTATTAATA ACTATGCTCA AAAATTGTGT
7800

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7860

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7920

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7980

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8040

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8100

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8160

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8220

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8280

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8340

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8400

GAAGCACTGT GGTTGCTGTG TTAGTAATGT GCAAAACAGG AGGCACATTT TCCCCACCTG
8460

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8520

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8580

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8640

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8700

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8760

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8820

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8880

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8940

CACTTTTCGG GGAAATGTGC GCGGAACCCC TATTGTTTA TTTTCTAAA TACATTCAAA
9000

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9060

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9120

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9180

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9240

CCCCGAAGAA CGTTTCCAA TGATGAGCAC TTTAAAGTT CTGCTATGTG GCGCGGTATT
9300

ATCCCGTGTT GACGCCGGC AAGAGCAACT CGGTCGCCGC ATACACTATT CTCAGAATGA
9360

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9420

ATTATGCAGT GCTGCCATAA CCATGAGTGA TAACACTGCG GCCAACTTAC TTCTGACAAC
9480

GATCGGAGGA CCGAAGGAGC TAACCGCTT TTTGCACAAC ATGGGGATC ATGTAACTCG
9540

CCTTGATCGT TGGGAACCGG AGCTGAATGA AGCCATACCA AACGACGAGC GTGACACCAC
9600

GATGCCTGCA GCAATGGCAA CAACGTTGCG CAAACTATTA ACTGGCGAAC TACTTACTCT
9660

AGCTTCCCGG CAACAATTAA TAGACTGGAT GGAGGCGGAT AAAGTTGCAG GACCACTTCT
9720

GCGCTCGGCC CTTCCGGCTG GCTGGTTAT TGCTGATAAA TCTGGAGCCG GTGAGCGTGG
9780

GTCTCGCGGT ATCATTGCAG CACTGGGCC AGATGGTAAG CCCTCCCGTA TCGTAGTTAT
9840

CTACACGACG GGGAGTCAGG CAACTATGGA TGAACGAAAT AGACAGATCG CTGAGATAGG
9900

TGCCTCACTG ATTAAGCATT GGTAACTGTC AGACCAAGTT TACTCATATA TACTTTAGAT
9960

TGATTTAAAA CTTCATTTT AATTTAAAAG GATCTAGGTG AAGATCCTTT TTGATAATCT
10020

CATGACCAAA ATCCCTTAAC GTGAGTTTC GTTCCACTGA GCGTCAGACC CCGTAGAAAA
10080

GATCAAAGGA TCTTCTTGAG ATCCTTTTT TCTGCGCGTA ATCTGCTGCT TGCAAACAAA
10140

AAAACCACCG CTACCAGCGG TGGTTTGTCTT GCCGGATCAA GAGCTACCAA CTCTTTTCC
10200

GAAAGTAACT GGCTTCAGCA GAGCGCAGAT ACCAAATACT GTCCTCTAG TGTAGCCGTA
10260

GTTAGGCCAC CACTCAAGA ACTCTGTAGC ACCGCCTACA TACCTCGCTC TGCTAATCCT
10320

GTTACCAGTG GCTGCTGCCA GTGGCGATAA GTCGTGTCTT ACCGGGTTGG ACTCAAGACG
10380

ATAGTTACCG GATAAGGCGC AGCGGTCGGG CTGAACGGGG GGTTCGTGCA CACAGCCCAG
10440

CTTGGAGCGA ACGACCTACA CCGAACTGAG ATACCTACAG CGTGAGCTAT GAGAAAGCGC
10500

CACGCTTCCC GAAGGGAGAA AGGCGGACAG GTATCCGGTA AGCGGCAGGG TCGGAACAGG
10560

AGAGCGCACG AGGGAGCTTC CAGGGGGAAA CGCCTGGTAT CTTTATAGTC CTGTCGGTT
10620

TCGCCACCTC TGACTTGAGC GTCGATTTTT GTGATGCTCG TCAGGGGGGC GGAGCCTATG
10680

GAAAAACGCC AGCAACGCCG CCTTTTTACG GTTCCTGGCC TTTTGCTGGC CTTTGCTCA
10740

CATGTTCTTT CCTGCGTTAT CCCCTGATTC TGTGGATAAC CGTATTACCG CCTTTGAGTG
10800

AGCTGATACC GCTCGCCGCA GCCGAACGAC CGAGCGCAGC GAGTCAGTGA GCGAGGAAGC
10860

- 111 -

GGAAGAGCGC CTGATGCGGT ATTTTCTCCT TACGCATCTG TGCAGTATTT CACACCGCAT
10920

ATGGTGCACT CTCAGTACAA TCTGCTCTGA TGCCGCATAG TTAAGCCAGT ATACACTCCG
10980

CTATCGCTAC GTGACTGGGT CATGGCTGCG CCCCCGACACC CGCCAACACC CGCTGACGCG
11040

CCCTGACGGG CTTGTCTGCT CCCGGCATCC GCTTACAGAC AAGCTGTGAC CGTCTCCGGG
11100

AGCTGCATGT GTCAGAGGTT TTCACCGTCA TCACCGAAC GCGCGAGGCA GC
11152

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GACGGATCGG GAGATCTCC

19

(2) INFORMATION FOR SEQ ID NO:14:

- 112 -

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCGCCTCAGA AGCCATAGAG CC

22

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14455 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAGCTTGGGC AGAAATGGTT GAACTCCGA GAGTGTCTA CACCTAGGGG AGAAGCAGCC

60

AAGGGGTTGT TTCCCACCAA GGACGACCCG TCTGCGCACA AACGGATGAG CCCATCAGAC

120

AAAGACATAT TCATTCTCTG CTGCAAAC TT GGCATAGCTC TGCTTGCCT GGGGCTATTG
180

GGGGAAGTTG CGGTTCGTGC TCGCAGGGCT CTCACCCCTG ACTCTTTAA TAGCTCTTCT
240

GTGCAAGATT ACAATCTAAA CAATTGGAG AACTCGACCT TCCTCCTGAG GCAAGGACCA
300

CAGCCAAC TT CCTCTTACAA GCCGCATCGA TTTTGTCTT CAGAAATAGA AATAAGAATG
360

CTTGCTAAAA ATTATATTT TACCAATAAG ACCAATCCAA TAGGTAGATT ATTAGTTACT
420

ATGTTAAGAA ATGAATCATT ATCTTTAGT ACTATTTTA CTCAAATTCA GAAGTTAGAA
480

ATGGGAATAG AAAATAGAAA GAGACGCTCA ACCTCAATTG AAGAACAGGT GCAAGGACTA
540

TTGACCACAG GCCTAGAAGT AAAAAAGGGA AAAAAGAGTG TTTTGTCAA AATAGGAGAC
600

AGGTGGTGGC AACCAAGGAC TTATAGGGGA CCTTACATCT ACAGACCAAC AGATGCC
660

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720

AAAGTGTAT ATAGATCCCT CCCTTTCGT GAAAGACTCG CCAGAGCTAG ACCTCCTTGG
780

TGTATGTTGT CTCAAGAAGA AAAAGACGAC ATGAAACAAC AGGTACATGA TTATATTTAT
840

CTAGGAACAG GAATGCACTT TTGGGGAAAG ATTTCCATA CCAAGGAGGG GACAGTGGCT
900

GGACTAATAG AACATTATTC TGCAAAA ACT CATGGCATGA GTTATTATGA ATAGCCTTTA
960

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1020

AACGAGGATG TGAGACAAAGT GGTTTCTGA CTTGGTTGG TATCAAAGGT TCTGATCTGA
1080

GCTCTGAGTG TTCTATTTTC CTATGTTCTT TTGGAATTAA TCCAAATCTT ATGTAATGC
1140

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1200

CATTCACCTC TTGTGTGTTT GTGTCTGTTC GCCATCCCGT CTCCGCTCGT CACTTATCCT
1260

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1320

GCTGGCGCCC GAACAGGGAC CCTCGGATAA GTGACCCCTG TCTCTATTTC TACTATTG
1380

TGTTTGTCTT GTATTGTCTC TTTCTTGTCT GGCTATCATC ACAAGAGCGG AACGGACTCA
1440

CCATAGGGAC CAAGCTAGCG ACTGAAAATG AGACATATTA TCTGCCACGG AGGTGTTATT
1500

ACCGAAGAAA TGGCCGCCAG TCTTTGGAC CAGCTGATCG AAGAGGTACT GGCTGATAAT
1560

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1620

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1680

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1740

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1800

ATGCCAAACC TTGTACCGGA GGTGATCGAT CTTACCTGCC ACGAGGCTGG CTTTCCACCC
1860

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1920

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1980

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2040

GTTGGTGATA GAGTGGTGGG TTTGGTGTGG TAATTTTTT TTTAATTTTT ACAGTTTGT
2100

GGTTTAAAGA ATTTGTATT GTGATTTTT TAAAAGGTCC TGTGTCTGAA CCTGAGCCTG
2160

AGCCCGAGCC AGAACCGGAG CCTGCAAGAC CTACCCGCCG TCCTAAAATG GCGCCTGCTA
2220

TCCTGAGACG CCCGACATCA CCTGTGTCTA GAGAATGCAA TAGTAGTACG GATAGCTGTG
2280

ACTCCGGTCC TTCTAACACA CCTCCTGAGA TACACCCGGT GGTCCCGCTG TGCCCCATTA
2340

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2400

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2460

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2520

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2580

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2640

TGGAAGATTT TTCTGCTGTG CGTAACCTGC TGGAACAGAG CTCTAACAGT ACCTCTTGGT
2700

TTTGGAGGTT TCTGTGGGC TCATCCCAGG CAAAGTTAGT CTGCAGAATT AAGGAGGATT
2760

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2820

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2880

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2940

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3000

TGAGACACAA GAATGCCCTG CTACTGTTGT CTTCCGTCCG CCCGGCGATA ATACCGACGG
3060

AGGAGCAGCA GCAGCAGCAG GAGGAAGCCA GGCGGCGCG GCAGGAGCAG AGCCCATGGA
3120

ACCCGAGAGC CGGCCTGGAC CCTCGGGAAT GAATGTTGTA CAGGTGGCTG AACTGTATCC
3180

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3240

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3300

CAGACACCGT CCTGAGTGTA TTACTTTCA ACAGATCAAG GATAATTGCG CTAATGAGCT
3360

TGATCTGCTG GCGCAGAAGT ATTCCATAGA GCAGCTGACC ACTTACTGGC TGCAGCCAGG
3420

GGATGATTT GAGGAGGCTA TTAGGGTATA TGCAAAGGTG GCACCTAGGC CAGATTGCAA
3480

GTACAAGATC AGCAAACCTG TAAATATCAG GAATTGTTGC TACATTCTG GGAACGGGGC
3540

CGAGGTGGAG ATAGATAACGG AGGATAGGGT GGCTTTAGA TGTAGCATGA TAAATATGTG
3600

GCCGGGGGTG CTTGGCATGG ACGGGGTGGT TATTATGAAT GTAAGGTTA CTGGCCCCAA
3660

TTTTAGCGGT ACGGTTTCC TGGCCAATAC CAACCTTATC CTACACGGTG TAAGCTTCTA
3720

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3780

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3840

CCTCTTGAA AGGTGTACCT TGGGTATCCT GTCTGAGGGT AACTCCAGGG TGCGCCACAA
3900

TGTGGCCTCC GACTGTGGTT GCTTCATGCT AGTGAAAAGC GTGGCTGTGA TTAAGCATAA
3960

CATGGTATGT GGCAACTGCG AGGACAGGGC CTCTCAGATG CTGACCTGCT CGGACGGCAA
4020

CTGTCACCTG CTGAAGACCA TTCACGTAGC CAGCCACTCT CGCAAGGCCT GGCCAGTGTT
4080

TGAGCATAAC ATACTGACCC GCTGTTCCCT GCATTTGGGT AACAGGAGGG GGGTGTTCCT
4140

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4200

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4380

CGCTGAGTTT GGCTCTAGCG ATGAAGATAC AGATTGAGGT ACTGAAATGT GTGGGCGTGG
4440

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4500

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4560

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4680

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5580

- 120 -

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6360

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6420

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6480

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6720

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6780

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6840

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6900

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6960

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7020

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7380

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7560

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7620

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7680

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8160

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8220

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8280

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8340

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8400

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8460

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8580

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8640

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8760

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8880

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9180

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9240

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9300

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9360

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9420

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9480

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9540

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9600

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9660

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9780

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9840

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9900

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10140

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10200

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10260

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10320

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10560

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10680

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10740

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10920

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10980

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11040

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11100

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12240

CCTTGTGAAT CAAAACCCAA GGCTCCAGTA ACTTTAGTTT GCAAGGAAGT ATTATTAATA
12300

GTCACACCTG GACCAGTTGC TACGGTCAAA GTGTTAGGT CGTCTGTTAC ATGCAAAGGA
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GCCCCGTACT TTAGTCCTAG TTTCCATTG TGTGTATAAA TGGGCTCTTT CAAGTCAATG
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12480

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12780

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12900

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12960

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13020

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13080

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13200

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13260

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13320

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- 130 -

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13440

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14280

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GGGAGCTGCA TGTGTCAGAG GTTTTCACCG TCATCACCGA AACGCGCGAG GCAGC
14455

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10610 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

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120

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180

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240

ACCACCACAT AGCTTATACA GATCACCGTA CCTTAATCAA ACTCACAGAA CCCTAGTATT
300

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420

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720

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780

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900

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960

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1380

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1440

AGTCATATTT CCTGAAGCAA AACCAAGGTGC GGGCGTGACA AACAGATCTG CGTCTCCGGT
1500

CTCGCCGCTT AGATCGCTCT GTGTAGTAGT TGTAGTATAT CCACTCTCTC AAAGCATCCA
1560

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1620

CCACCACCGC AGAATAAGCC ACACCCAGCC AACCTACACA TTCGTTCTGC GAGTCACACA
1680

CGGGAGGAGC GGGAAAGAGCT GGAAGAACCA TGTTTTTTT TTTATTCCAA AAGATTATCC
1740

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1800

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1860

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1920

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1980

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2040

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2100

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2160

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2220

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2280

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2340

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2400

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2460

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2580

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2640

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2700

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2760

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2820

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2880

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2940

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3000

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3060

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3120

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3180

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3240

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3300

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3360

CCAGATATAC GCGTTGACAT TGATTATTGA CTAGTTATTA ATAGTAATCA ATTACGGGGT
3420

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3480

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3720

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3780

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CCCCATTGAC GCAAATGGGC GGTAGGCGTG TACGGTGGGA GGTCTATATA AGCAGAGCTC
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4140

CGAACGGTAC TCCGCCACCG AGGGACCTGA GCGAGTCCGC ATCGACCGGA TCGGAAAACC
4200

TCTCGAGAAA GGC GTCTAAC CAGTCACAGT CGCAAGGTAG GCTGAGCACC GTGGCGGGCG
4260

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4500

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4560

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9300

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10320

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10500

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(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

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(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

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35

G

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

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(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 amino acids
 - (B) TYPE: amino acid
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- (ii) MOLECULE TYPE: peptide
- (v) FRAGMENT TYPE: internal

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1									10		

We claim:

1. A packaging cell line expressing one or more adenovirus structural proteins, polypeptides, or fragments thereof, wherein said structural protein is selected from the group consisting of:
 - a. penton base;
 - b. hexon;
 - c. fiber;
 - d. polypeptide IIIa;
 - e. polypeptide V;
 - f. polypeptide VI;
 - g. polypeptide VII;
 - h. polypeptide VIII; and
 - i. biologically active fragments thereof.
2. A packaging cell line according to claim 1, which supports the production of a viral vector.
3. A packaging cell line according to claim 2, wherein the viral vector comprises a tripartite leader sequence or a sequence substantially homologous thereto.
4. A packaging cell line according to any one of the preceding claims wherein said structural protein is fiber and wherein said fiber protein has been modified to include a non-native amino acid residue sequence which targets a specific receptor, but which does not disrupt trimer formation or transport of fiber into the nucleus.
5. A packaging cell line according to claim 4 wherein said non-native amino acid residue sequence alters the binding specificity of the fiber for a targeted cell type.
6. A packaging cell line according to any one of the preceding claims wherein said structural protein is fiber comprising amino acid residue sequences from more than one adenovirus serotype.
7. A packaging cell line according to any one of claims 2 to 6, wherein said viral vector includes a nucleic acid sequence having a deletion or mutation of a DNA sequence encoding an adenovirus structural protein, polypeptide, or fragment thereof.

8. A packaging cell line according to claim 7 wherein said viral vector includes a nucleic acid sequence having a deletion or mutation of the DNA sequences encoding regulatory polypeptides E1A and E1B.
9. A packaging cell line according to claim 7, wherein said viral vector further includes a nucleic acid sequence having a deletion or mutation of a DNA sequence encoding one or more of the following regulatory proteins or polypeptides: E2A, E2B, E3, E4, L4, or fragments thereof.
10. A packaging cell line according to claim 7, wherein a foreign DNA sequence encoding one or more foreign proteins, polypeptides or fragments thereof has been inserted in place of any of said deletions in said therapeutic viral vector.
11. A packaging cell line according to claim 10, wherein said foreign DNA encodes a tumor-suppressor protein or a biologically active fragment thereof.
12. A packaging cell line according to claim 10, wherein said foreign DNA encodes a suicide protein or a biologically active fragment thereof.
13. A packaging cell line according to any one of the preceding claims, wherein said cell line is an epithelial cell line.
14. A packaging cell line according to claim 12, wherein said cell line is selected from the group consisting of 293, A549, W162, HeLa, Vero, 211, and 211A cell lines.
15. A viral vector comprising deletion or mutation of a DNA sequence encoding an adenovirus structural protein, polypeptide, or fragment thereof, wherein said structural protein, polypeptide or fragment thereof is selected from the group consisting of:
 - a. penton base;
 - b. hexon;
 - c. fiber;
 - d. polypeptide IIIa;
 - e. polypeptide V;
 - f. polypeptide VI;
 - g. polypeptide VII;
 - h. polypeptide VIII; and
 - i. biologically active fragments thereof.

16. A viral vector according to claim 15, further comprising deletion or mutation of the DNA sequences encoding regulatory polypeptides E1A and E1B, or fragments thereof.
17. A viral vector according to claim 15, further comprising deletion or mutation of the DNA sequence encoding one or more of the following regulatory proteins or polypeptides: E2A, E2B, E3, E4, L4, or fragments thereof.
18. A viral vector according to claim 15 further comprising a foreign DNA sequence inserted in place of the DNA sequence encoding said structural protein, polypeptide, or fragment thereof.
19. A viral vector according to any one of claims 16, 17 or 18, further comprising foreign DNA sequences inserted in place of the DNA sequences encoding said regulatory polypeptides or fragments thereof.
20. A viral vector lacking all or part of a DNA sequence encoding adenovirus fiber protein, wherein said DNA sequence has been replaced by a foreign DNA sequence encoding a therapeutic molecule.
21. A viral vector having a mutation in a DNA sequence encoding adenovirus fiber protein.
22. A viral vector according to any one of 15 to 21 wherein the vector is a therapeutic vector.
23. A complementing plasmid comprising a promoter nucleotide sequence operatively linked to a nucleotide sequence encoding an adenovirus structural protein, polypeptide, or fragment thereof.
24. A complementing plasmid according to claim 23, further comprising a nucleotide sequence encoding:
 - a. a first adenovirus regulatory protein, polypeptide, or fragment thereof; or
 - b. a second regulatory protein, polypeptide, or fragment thereof; or
 - c. a third regulatory protein, polypeptide, or fragment thereof; or
 - d. any combination of the foregoing.
25. A complementing plasmid according to claim 23, wherein said adenovirus structural protein or polypeptide is selected from the group consisting of:
 - a. penton base;

- b. hexon;
- c. fiber;
- d. polypeptide IIIa;
- e. polypeptide V;
- f. polypeptide VI;
- g. polypeptide VII;
- h. polypeptide VIII; and
- i. biologically active fragments thereof.

26. A complementing plasmid according to claim 24, wherein said regulatory proteins, polypeptides or fragments thereof are selected from the group consisting of E1A, E1B, E2A, E2B, E3, E4, and L4.

27. A complementing plasmid comprising a promoter nucleotide sequence operatively linked to a nucleotide sequence encoding an adenovirus regulatory protein, polypeptide, or fragment thereof.

28. A composition useful in the preparation of a therapeutic viral vectors, the composition comprising a cell containing a delivery plasmid comprising an adenovirus genome lacking a nucleotide sequence encoding fiber.

29. A composition according to claim 28, wherein said delivery plasmid further comprises a nucleotide sequence encoding a foreign polypeptide.

30. A composition according to claim 29, wherein said polypeptide is a therapeutic molecule.

31. A composition according to claim 28, wherein said delivery plasmid is selected from the group consisting of pDV1, p E1B gal, p E1sp1B, and pFG140-f.

32. A composition according to claim 28, wherein said cell further comprises a complementing plasmid containing a nucleotide sequence encoding fiber, said plasmid being stably integrated into the cellular genome of said cell.

33. A composition according to claim 32, wherein said complementing plasmid has the characteristics of pCLF having ATCC Accession Number 97737.

34. A composition useful in the preparation of therapeutic viral vectors, said composition comprising a cell containing:

- a. a first delivery plasmid comprising an adenovirus genome lacking a nucleotide sequence encoding fiber and incapable of directing the packaging of new viral particles in the absence of a second delivery plasmid; and
- b. a second delivery plasmid comprising an adenoviral genome capable of directing the packaging of new viral particles in the presence of said first delivery plasmid.
35. A composition according to claim 34, wherein said first and second delivery plasmids interact within said cell to produce a therapeutic viral vector.
36. A composition according to claim 34, wherein said cell further comprises a complementing plasmid containing a nucleotide sequence encoding fiber, said plasmid being stably integrated into the cellular genome of said cell.
37. A composition according to claim 34, wherein said first or second delivery plasmid further comprises a nucleotide sequence encoding a foreign polypeptide.
38. A composition according to claim 37, wherein said polypeptide is a therapeutic molecule.
39. A composition according to claim 34, wherein said first delivery plasmid lacks adenovirus packaging signal sequences.
40. A composition according to claim 34, wherein said second delivery plasmid contains a LacZ reporter construct.
41. A composition according to claim 34, wherein said second delivery plasmid further lacks a nucleotide sequence encoding an adenovirus regulatory protein.
42. A composition according to claim 41, wherein said regulatory protein is E1.
43. A composition according to claim 36, wherein said complementing plasmid has the characteristics of pCLF having ATCC Accession Number 97737.
44. A composition according to claim 34, wherein said first delivery plasmid lacks a nucleotide sequence encoding adenovirus E4 protein and said second delivery plasmid lacks a nucleotide sequence encoding adenovirus E1 protein.
45. A composition according to claim 44, wherein said cell contains at least one complementing plasmid encoding an adenoviral regulatory protein and a structural protein.
46. A composition according to claim 45, wherein said regulatory protein is E4 and said structural protein is fiber.

47. A composition according to claim 45, wherein said regulatory protein is E1 and said structural protein is fiber.
48. A composition according to claim 45, wherein said regulatory protein is both E1 and E4 and said structural protein is fiber.
49. A composition according to claim 45, wherein said adenoviral regulatory protein and said structural protein are encoded by separate complementing plasmids.
50. A composition according to claim 45, wherein said cell is selected from the group consisting of 293, A549, W162, HeLa, Vero, 211, and 211A.

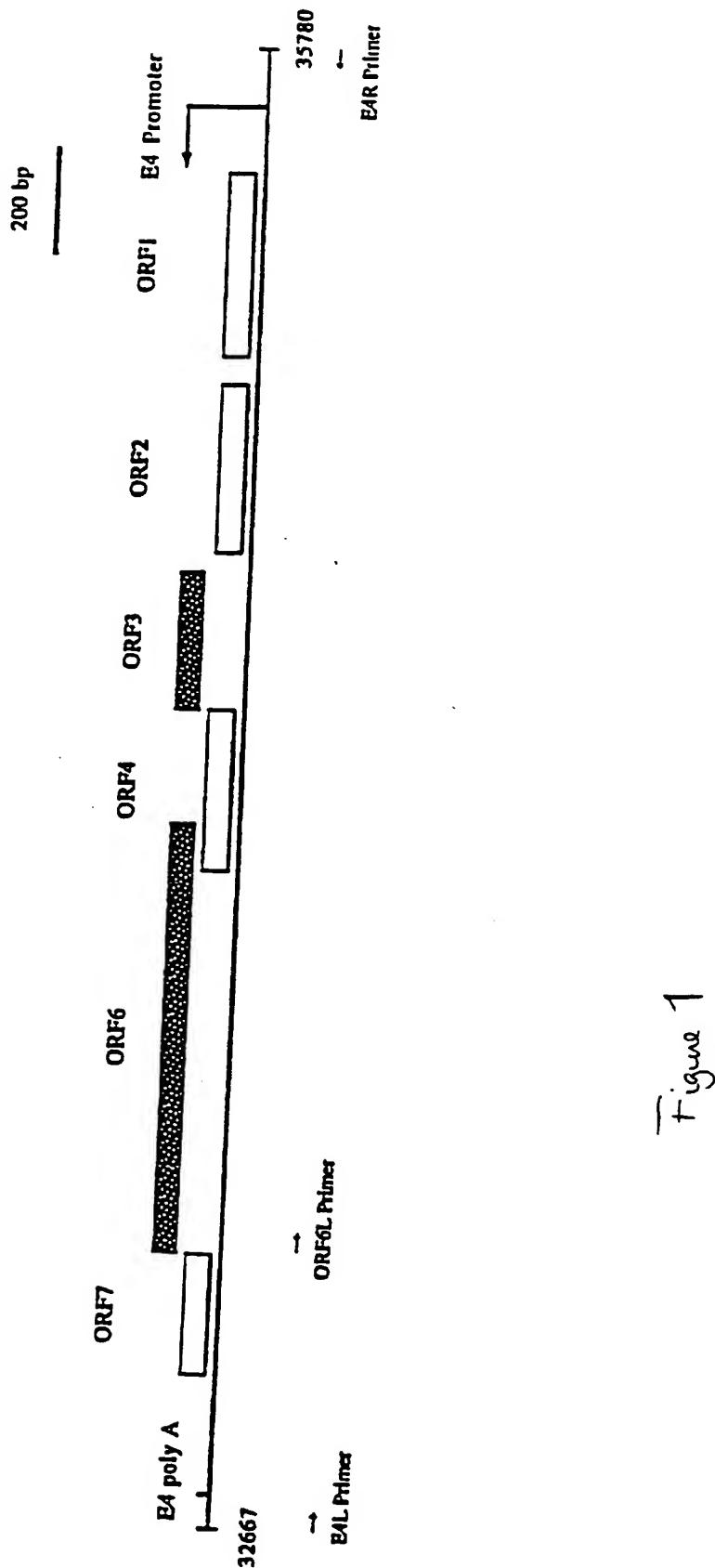


Figure 1

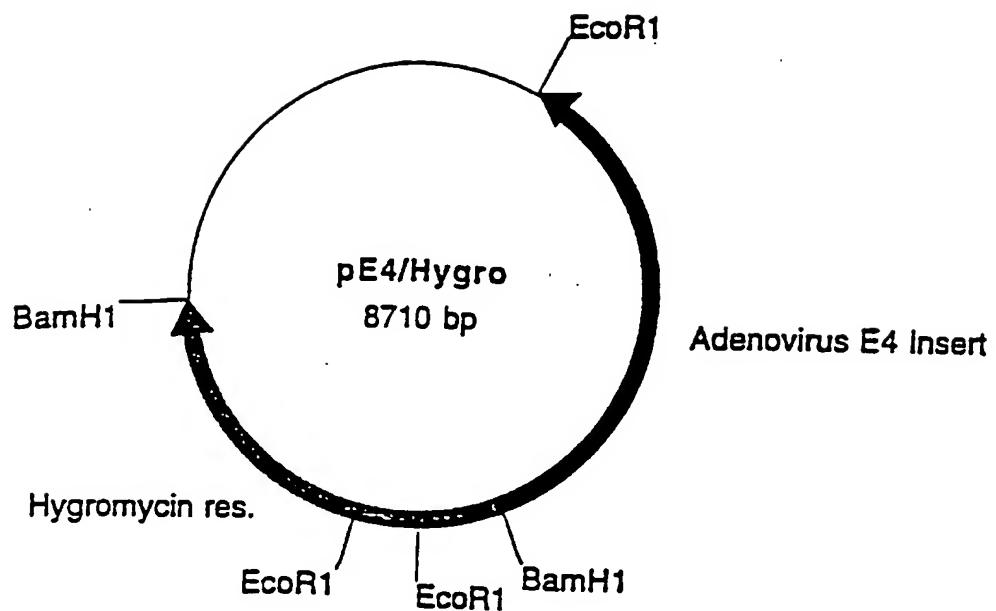


Figure 2

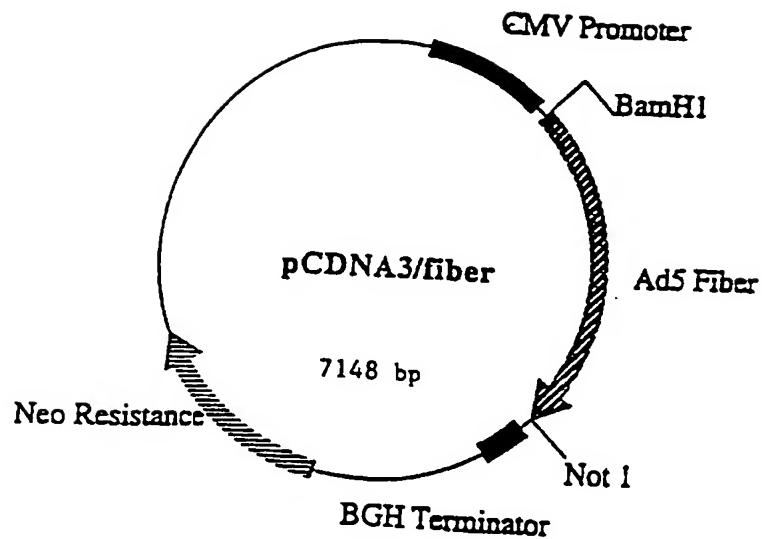


FIGURE 3

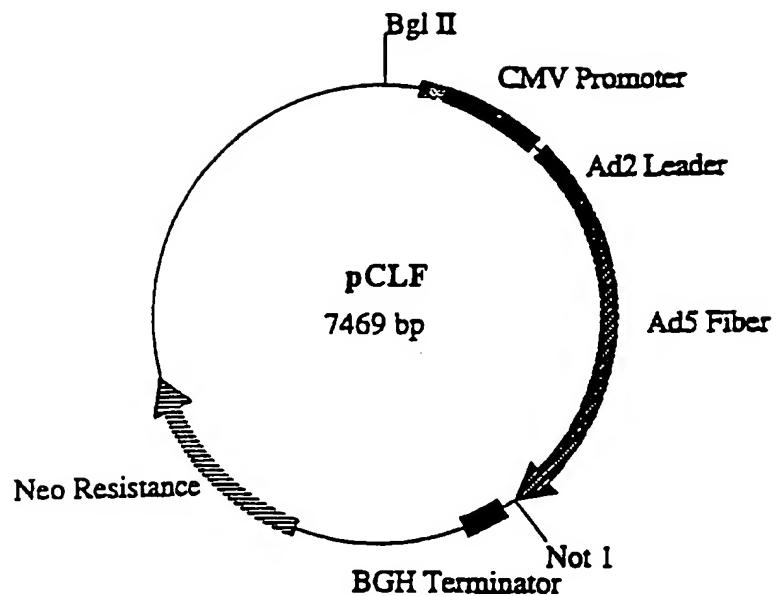


Figure 4

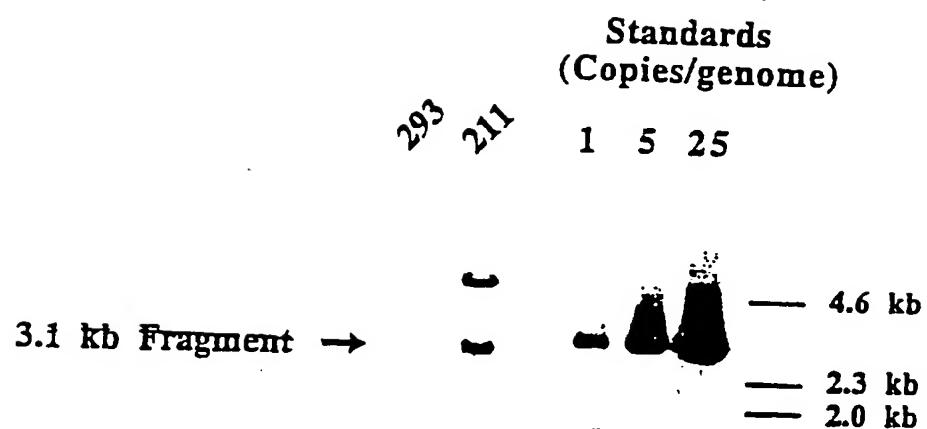


Figure 5

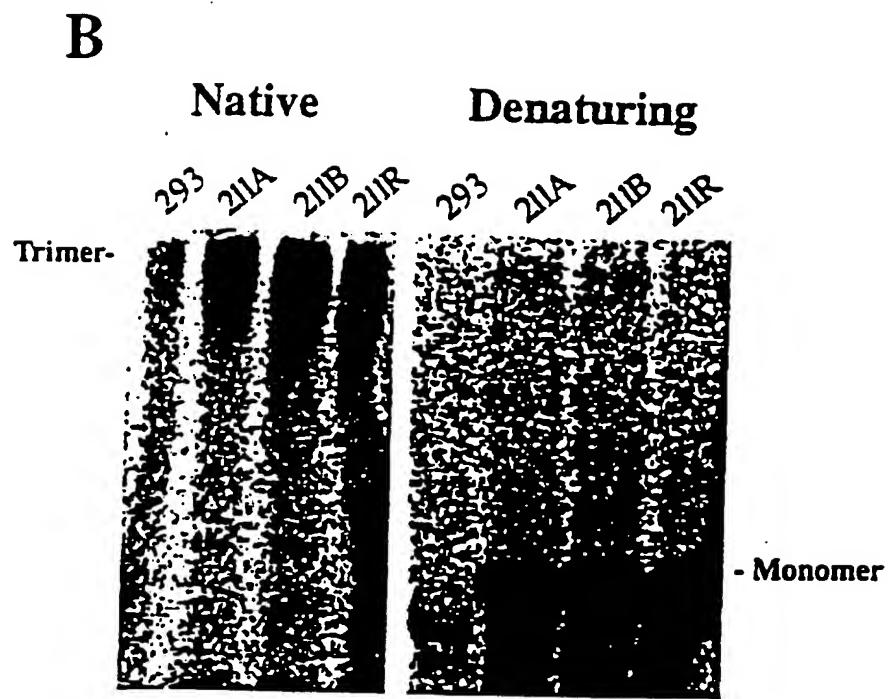


Figure 6

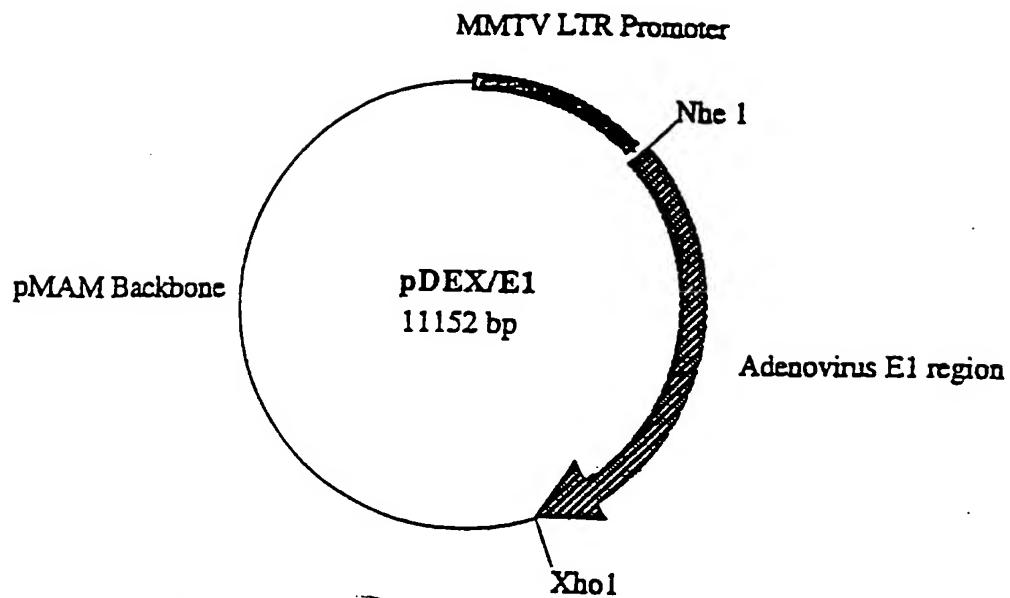


Figure 7

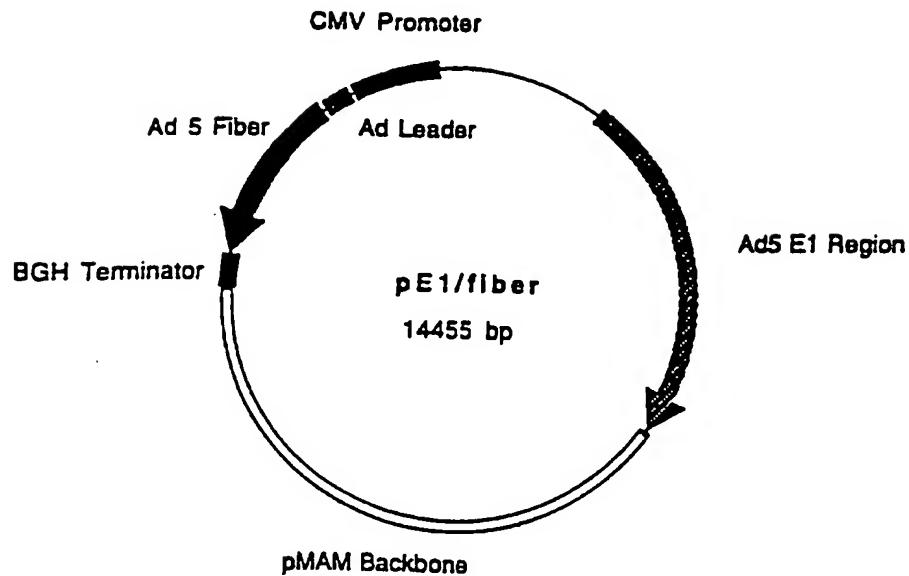


Figure 8

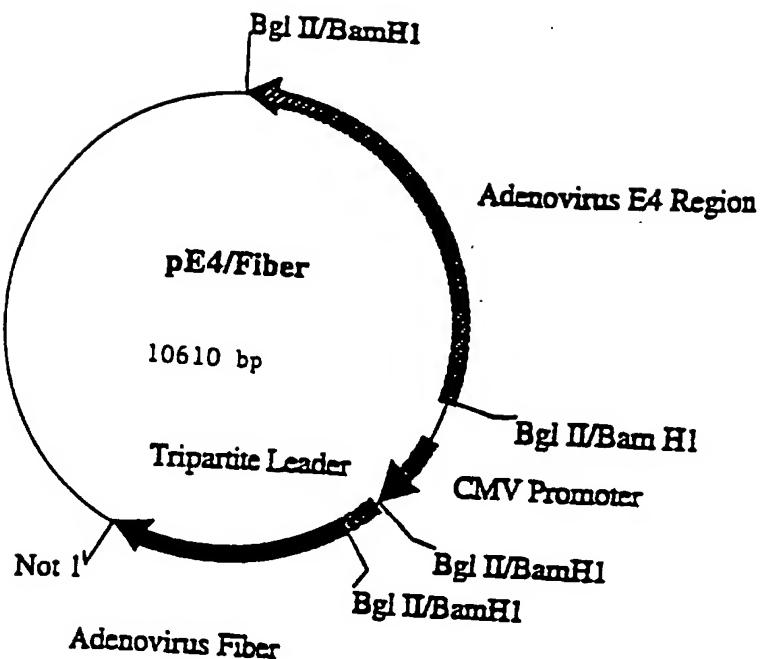


Figure 9



FIGURE 10

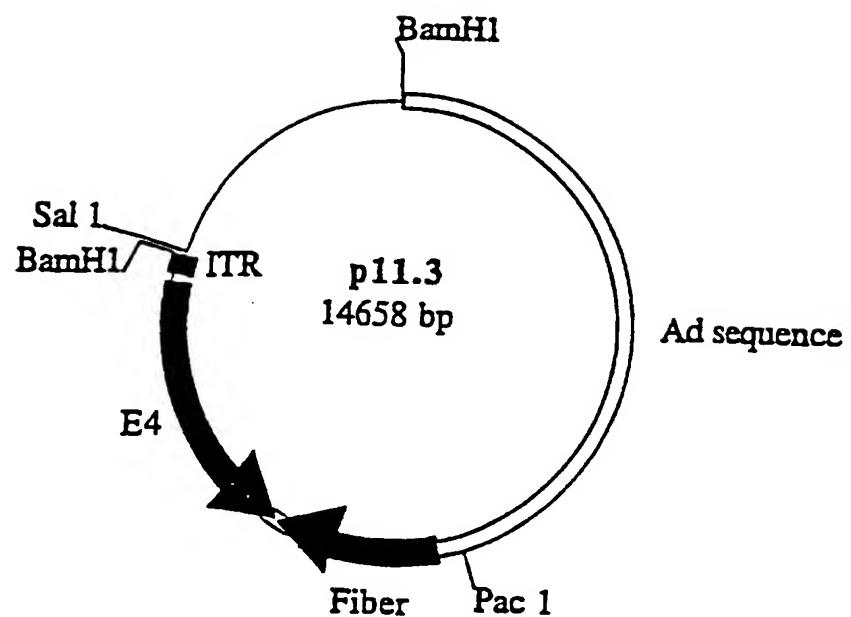


Figure 11

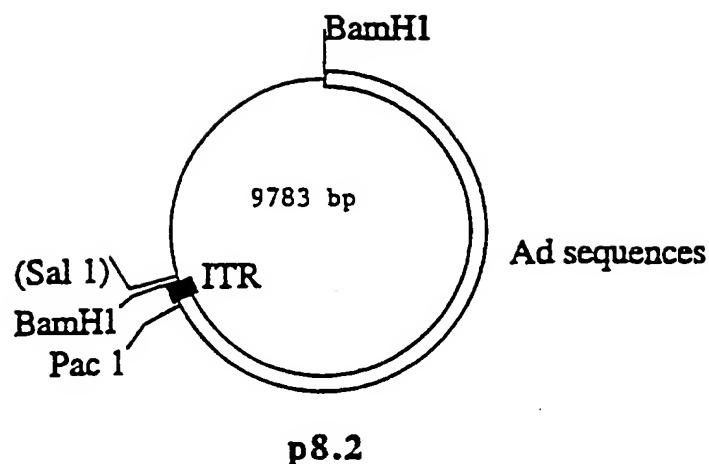


FIGURE 12

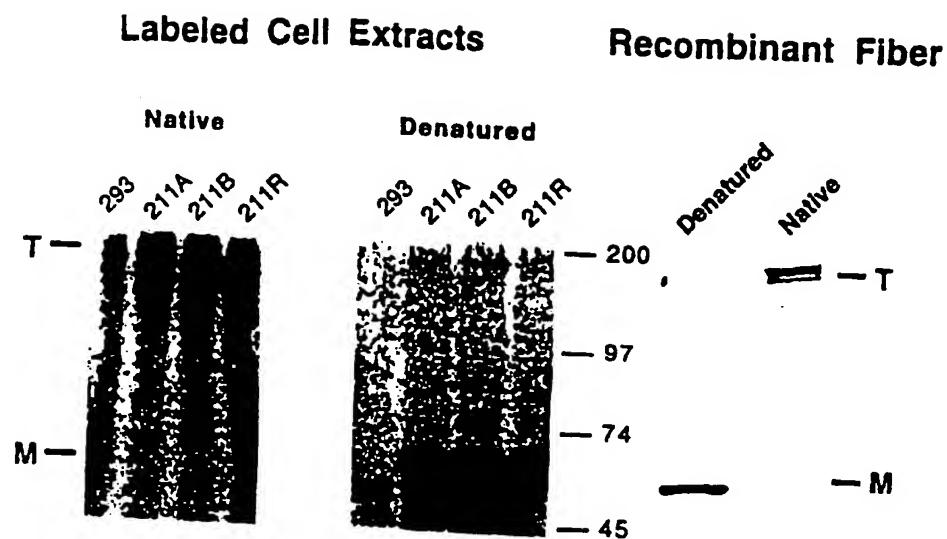


Figure 13

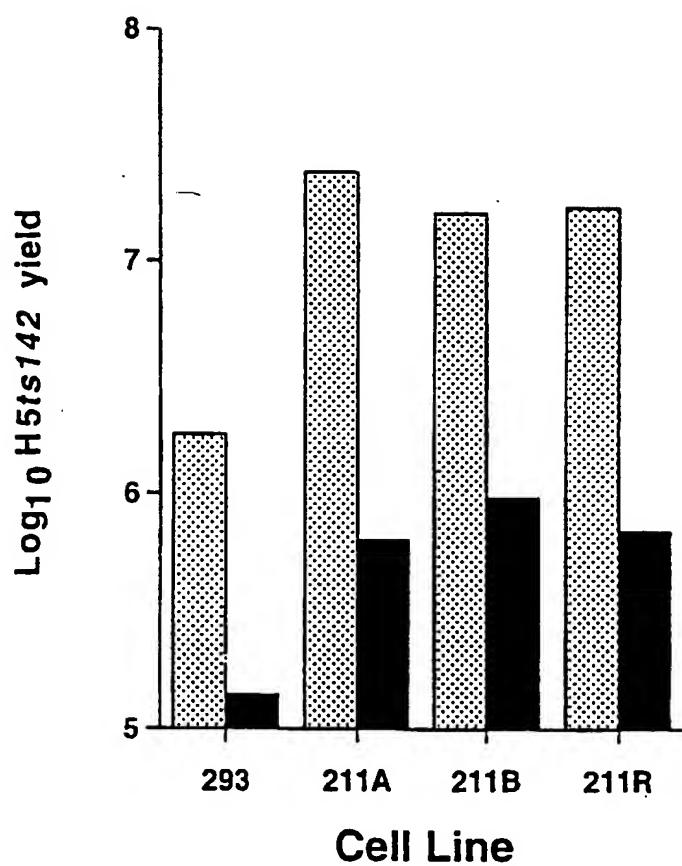


Figure 14

A

Ad2/5 M K R A R P S E D T F N P V Y P Y D T E T G P P T V P F L T P P F V S P N G F O E S P P
 Ad3 M A K R A R L S - T S F N P V Y P Y E D E S S S - Q H P F I N P G F I S P D G F T O S P N
 Ad19/37 M S K R L R V E D D F N P V Y P Y G Y A R N O - N I P F L T P P F V S B D G F K N F P P
 Ad40 (1) M - K R A R F - E D D F N P V Y P Y E H Y M - P L D I P F I T P P F A S S N G L O E K P P
 Ad40 (2) M - K R T R - I E D D F N P V Y P Y D T S S T P S - I P Y V A P P F V S B D G L O E N P P

B

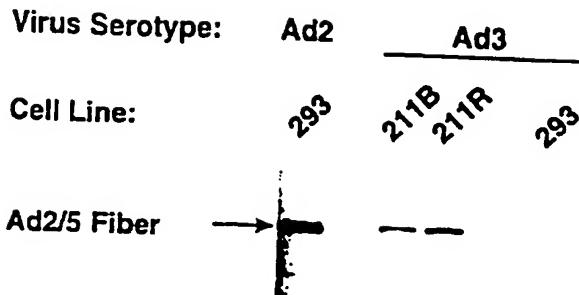


Figure 15

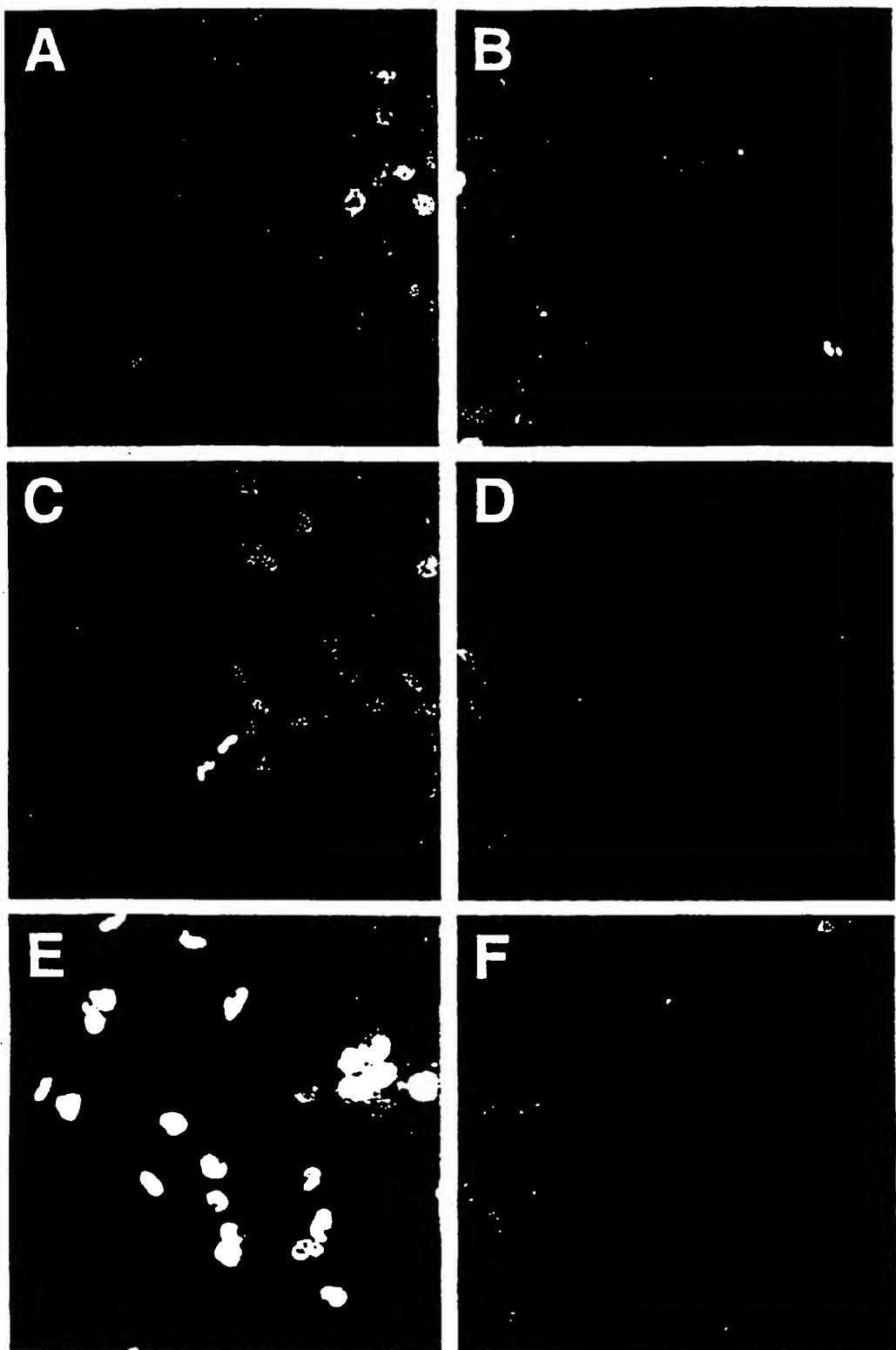


Figure 1b

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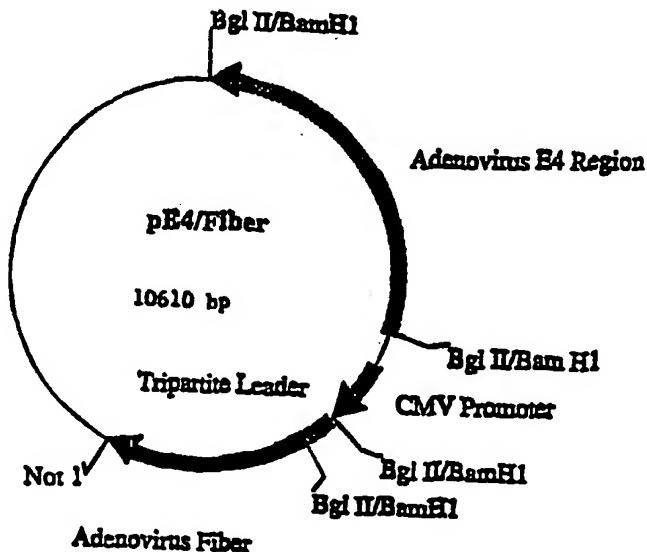
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(54) Title: **PACKAGING CELL LINES FOR USE IN FACILITATING THE DEVELOPMENT OF HIGH-CAPACITY ADENOVIRAL VECTORS**



(57) Abstract

The present invention relates to gene therapy, especially to adenovirus-based gene therapy, and related cell lines and compositions. In particular, novel packaging cell lines are disclosed, for use in facilitating the development of high-capacity vectors. The invention also discloses a variety of high-capacity adenovirus vectors and related compositions and kits including the disclosed cell lines and vectors. Finally, the invention discloses methods of preparing and using the disclosed vectors, cell lines and kits.

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